University of Alberta

Characterization and Regulation of the Murine Triacylglycerol Hydrolase

by

Vernon Wayne Dolinsky

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the

requirements for the degree of Doctor of Philosophy

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DEDICATION

To Mom and Dad

Thank you for your encouragement and support

throughout the many years of school.

ABSTRACT

Stored intracellular triacylglycerol (TG) undergoes lipolysis followed by re-esterification prior to its assembly into a very low density lipoprotein (VLDL) particle. Triacylglycerol hydrolase (TGH) is a lipase that is involved in the lipolysis of hepatic intracellular stored TG. A novel cDNA encoding murine TGH was cloned and characterized. The murine TGH is structurally similar to other lipases and has close sequence identity to carboxylesterases, including conserved serine, glutamate and histidine catalytic residues. Murine TGH is highly expressed in liver as well as heart, kidney, small intestine and adipose tissues. The murine TGH gene spans 35kb, is located on chromosome 8 and is composed of 14 small exons and 13 introns. The murine TGH promoter requires the transcription factor Sp1 for basal activity. A vector for the targeted deletion of the murine TGH gene has been cloned. Murine TGH expression is hormonally regulated. Dexamethasone suppressed hepatic TGH expression and activity, as well as the lipolysis of intracellular stored TG by primary murine hepatocytes. Dexamethasone reduced the post-transcriptional stability of TGH mRNA. TGH expression was absent in liver and adipose tissues

from obese rodents. Evidence for a possible role for TGH in basal lipolysis and leptin-stimulated lipolysis in adipocytes is presented.

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List of Abbreviations

AADA	Arylacetamide deaceytlase
ACAT	Acyl-CoA :Cholesterol acyltransferase
ACTH	adrenocorticotropic hormone
ADRP	Adipocyte differentiation related protein
AGPAT	1-acylglycerol-3-phosphate acyltransferase
ALBP	adipocyte lipid binding protein
AMP	adenosine monophosphate
aP2	adipocyte fatty acid binding protein
apoB	apoprotein B
BAC	bacterial artificial chromosome
cAMP	cyclic adenosine monophoshate
cDNA	complementary deoxyribonucleic acid
CE	cholesteryl ester
CHO	Chinese hamster ovary
CRH	corticotropin releasing hormone
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dNTPs	deoxyribonucleotide triphosphates
dex	dexamethasone
DG	diacylglycerol
DGAT	diacylglycerol acyltransferase
DGTA	diacylglycerol transacylase
DMEM	Dulbecco's modified Eagle's medium
E600	diethyl-*p*-nitrophenyl phosphate
ER	endoplasmic reticulum
Es 22	esterase 22/egasyn

Es X	esterase X
FA	fatty acid
FAT	fatty acid transporter
FATP	fatty acid transport protein
GPAT	glycerol-3-phosphate acyltransferase
HPA	hypothalamic-pituitary-adrenal axis
HSL	hormone sensitive lipase
LDL	low density lipoprotein
MG	monoacylglycerol
MGAT	monoacylglycerol acyltransferase
MGL	monoacylglycerol lipase
mRNA	messenger ribonucleic acid
MTP	microsomal triglyceride transfer protein
MUH	4-methylumbelliferyl heptanoate
NEFA	non-esterified fatty acid
PA	phosphatidic acid
PAPH	phosphatidate phosphohydrolase
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
PKA	protein kinase A
PPAR	peroxisomal proliferators activated receptor
RACE	rapid amplification of cDNA ends
rNTPs	ribonucleotide triphosphates
SCD	stearoyl-CoA desaturase
SDS	sodium dodecyl sulphate
SRE	sterol response element
SREBP	sterol response element binding protein
TG	triacylglycerol

TGH	triacylglycerol hydrolase
TLC	thin layer chromatography
tRNA	transfer ribonucleic acid
VLDL	very low density lipoprotein

CHAPTER 1

INTRODUCTION

1. INTRODUCTION TO THE MAINTENANCE OF ENERGY BALANCE AND THE MICROSOMAL TRIACYLGLYCEROL HYDROLASE.

Triacylglycerol (TG) is the most concentrated form of energy in biological tissues. TG is composed of a glycerol backbone to which three fatty acyl chains are attached via an ester linkage. Excessive storage of TG occurs when total energy intake exceeds total body energy expenditure. The sedentary lifestyle and high fat diets of our western world have resulted in the increased prevalence of obesity. Obesity may be defined as a state of increased body weight, more specifically of adipose tissue, of sufficient magnitude to produce adverse metabolic consequences. Obesity is associated with type-II diabetes, hyperlipidemia and cardiovascular disease (1-3). Excessive TG storage has a lipotoxic effect and predisposes tissues to insulin resistance leading ultimately to severe diabetes (4). Excessive hepatic TG secretion contributes to high levels of circulating apolipoprotein B and TG (5). These factors pre-dispose individuals to coronary heart disease (5). Therefore, excessive levels of tissue or circulating TGs have negative effects on an individual. If the balance between TG synthesis and storage versus TG hydrolysis and fatty acid oxidation is disturbed, the development of obesity and atherosclerosis is likely.

A lipase, termed triacylglycerol hydrolase (TGH) that functions to mobilize stored TG was purified from porcine liver microsomes (6). TGH accounts for approximately 70% of hepatic microsomal alkaline lipolysis (7). Structurally, TGH is related to the

carboxylesterase gene family (8) and has an α/β hydrolase fold, characteristic of all lipases (9). TGH hydrolyzes stored TG within McArdle RH7777 hepatoma cells and the lipolytic products are directed into the secretory pathway as judged by the increased VLDL-TG and apolipoprotein B secreted by these cells (10). Furthermore, inhibition of TGH reduced TG and apolipoprotein B secretion both *in vitro* (7) and *in vivo* (11). Therefore a role for TGH as a regulator of the provision of stored TG for hepatic VLDL secretion is suggested (7, 10, 11). TGH is also expressed in adipose tissue (8). Given that the targeted deletion of hormone sensitive lipase (HSL) failed to eliminate the lipolysis of stored TG within adipose tissue (12, 13), a potential role for TGH in the mobilization of the TG stored within adipose tissue exists. Indeed, inhibition of TGH reduced adipose tissue free fatty acid release both *in vitro* (14) and *in vivo* (11).

Investigations into the function of TGH and its potential regulation could identify a link between TGH and the maintenance of the correct energy balance. This could provide the basis for future therapeutic approaches that may ameliorate the risk of atherosclerosis and insulin resistance. Therefore, by way of introduction, the pathways of TG biosynthesis, storage and secretion, as well as their regulation are reviewed. Then, the regulation and mechanisms for the lipolysis of stored TG are reviewed with special attention given to the role of TG lipolysis and re-esterification in hepatic VLDL-TG secretion as well as adipose tissue metabolism. A review of the role for TGH in these processes will be detailed where appropriate.

1.1 TRIACYLGLYCEROL BIOSYNTHESIS

Variations in the composition and quantity of the diet affect the rate of *de novo* fatty acid and TG synthesis to meet the body's requirement for lipid and energy storage. The source of the fatty acids for TG synthesis varies depending upon the tissue examined. For example, the enterocyte takes up dietary fatty acids and 2-monacylglycerols (MG) and resynthesizes TG from these substrates primarily through the 2-monoacylglycerol pathway (see 1.3). Hepatocytes take up fatty acids derived from remnant lipoproteins, free fatty acids released from adipocytes and when in the fed state, a high carbohydrate diet stimulates the synthesis of fatty acids from carbohydrates. It is generally accepted that the synthesis of TG is controlled primarily by the amount of fatty acid available. Thus, *de novo* TG synthesis is enhanced only when fatty acid synthesis or uptake exceeds the cellular need for energy and phospholipid synthesis. In hepatocytes, as well as adipocytes, *de novo* TG synthesis is primarily achieved through the glycerol phosphate pathway (see 1.2).

1.2 GLYCEROL PHOSPHATE PATHWAY

The glycerol phosphate pathway is the main route of TG biosynthesis in non-enteric tissues (Figure 1.1). Briefly, it begins with the acylation of glycerol-3-phosphate by glycerol-3-phosphate acyltransferase (GPAT) at the *sn*-1 position, to form 1-acylglycerol-3-phosphate. Subsequently, the acylation of 1-acylglycerol-3-phosphate at the *sn*-2 position by 1-acylglycerol-3-phosphate acyltransferase (AGPAT) (also referred to as lysophosphatidic acid acyltransferase) synthesizes phosphatidic acid (PA). The hydrolysis of the sn-3 phosphate by phosphatidate phosphohydrolase (PAPH) generates 1,2-diacylglycerol (DG). DG is then acylated to form TG via the action of diacylglycerol acyltransferase (DGAT). The utilization of molecular cloning and gene targeting techniques has elucidated many new insights regarding these enzymes and their regulation. The regulation of TG synthesis and its breakdown determines the quantity of TG stored within a cell.

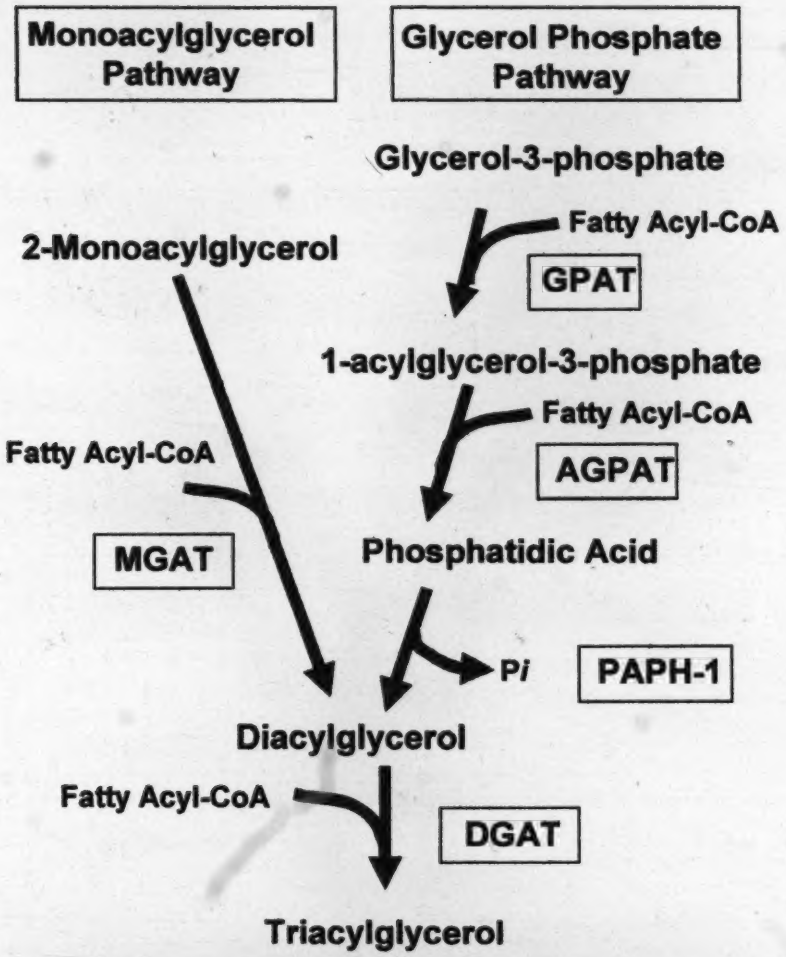


Figure 1.1- The two major pathways for triacylglycerol synthesis. In the monoacylglycerol pathway, monoacylglycerol acyltransferase (MGAT) produces diacylglycerol by joining a fatty acyl group to 2-monoacylglycerol. In the glycerol phosphate pathway, the acylation of glycerol-3-phosphate by glycerol-3-phosphate acyltransferase (GPAT) and acylation of 1-acyl-glycerol-3-phosphate by 1-acyl-glycerol-3-phosphate acyltransferase (AGPAT) produces phosphatidic acid. Hydrolysis of the sn-3 phosphate through the action of microsomal phosphatidate phosphohydrolase (PAPH-1) produces diacylglycerol. Diacylglycerol may be used subsequently for the production of triacylglycerol by diacylglycerol acyltransferase (DGAT).

1.2.1 *Glycerol-3-phosphate acyltransferase* (EC 2.3.1.15)

GPAT represents the committed step for the synthesis of all glycerolipids. Two isoforms for this enzyme exist. A mitochondrial enzyme that has been cloned and demonstrated to be tightly regulated (15). A microsomal GPAT activity also exists though a mammalian cDNA has not been cloned and its activity appears to be largely unregulated (15). The murine mitochondrial GPAT cDNA is primarily expressed in liver, adipose and muscle tissues (16). Interestingly, discordance between mitochondrial GPAT mRNA levels, protein expression and activities was observed in a variety of rat tissues (17). This observation suggests that GPAT expression is uniquely regulated in each tissue, possibly due to the unique needs of each tissue for TG or phospholipid synthesis.

The nutritional and hormonal regulation of the murine mitochondrial GPAT is well characterized and occurs primarily at the transcriptional level. Post-translational reduction of mitochondria GPAT activity has been reported to occur upon stimulation of AMP-activated kinase, a sensor of cellular fuel deprivation (18). In liver and adipose tissue, murine GPAT mRNA is decreased during fasting and induced after refeeding a high carbohydrate, low fat diet (19). Changes in nutrient intake lead to changes in circulating glucose which signal the secretion of hormones. For example, glucagon is elevated during starvation, while insulin is elevated during ingestion of high carbohydrate diet. The effect of streptozotocin-induced diabetes in the mouse is to reduce GPAT mRNA levels, while insulin replacement dramatically stimulated GPAT mRNA levels (20). Administration of glucagon to fasted mice during refeeding blocked the previously observed induction of GPAT

mRNA (20). Furthermore, obese Zucker rats, that have a defective leptin receptor and are insulin resistant, exhibit enhanced GPAT activity in both hepatic and adipose tissues (21). *In vitro*, overexpression of active sterol regulatory element binding protein (SREBP) stimulated GPAT promoter activity and SREBP bound an SRE-element within the GPAT proximal promoter, though feeding mice sterol enriched diets failed to significantly change the expression of GPAT mRNA in the liver (22). Therefore, the regulation of mitochondrial GPAT is consistent with the need to reduce TG synthesis and increase oxidation when nutrients are limiting, and direct FA toward TG synthesis and away from oxidation when energy is in excess.

Mitochondrial GPAT is composed of two transmembrane domains with the active site directed towards the cytosol (23). The localization of a highly regulated GPAT enzymatic activity at the cytosolic surface of the mitochondria suggests that this activity is important to prevent fatty acyl-CoA from undergoing beta-oxidation under certain conditions by converting it to 1-acylglycerol-3-phosphate and directing the FA toward TG synthesis. When the mitochondrial GPAT cDNA was over expressed in CHO cells and HEK293 cells, which normally contain minimal GPAT enzyme, exogenously provided fatty acids were preferentially directed towards TG synthesis, but not phospholipids (24). This result suggested that the 1-acylglycerol-3-phosphate produced remains separate from the pool that contributed to the synthesis of phospholipids.

1.2.2 *1-Acylglycerol-3-phosphate acyltransferase* (EC 2.3.1.51)

Several AGPAT cDNA sequences have been identified. Initially, human and mouse cDNAs of 2kb and 1.8 kb respectively, that encoded a protein having AGPAT activity were identified and designated AGPAT-1 (25, 26). AGPAT-1 overexpression in 3T3-L1 adipocytes inhibited NEFA release and promoted TG storage and FA uptake (27). Ubiquitous expression of AGPAT-1 in all tissues examined, with highest expression in skeletal muscle, suggested that it is a housekeeping gene (25, 26). AGPAT-2 cDNA is expressed at highest levels in the liver, heart and adipose tissues and much less in other tissues (28, 29). The protein encoded by the AGPAT-2 cDNA shares 48% homology with AGPAT-1 and is expressed at two-fold higher levels than AGPAT-1 in the adipose tissue, similar levels in liver and AGPAT-1 is expressed at 1.8-fold greater levels than AGPAT-2 in skeletal muscle (30). Recently, several individuals having congenital generalized lipodystrophy were discovered to have a variety of different mutations in the AGPAT2 gene leading to the production of a dysfunctional protein (30). These observations suggested that AGPAT2 was necessary for TG synthesis in adipocytes. Three additional AGPAT cDNAs have been designated AGPAT-3,4,5 respectively. These three cDNAs are barely detectable in liver, adipose and skeletal muscle and currently do not have a clearly identified function (30). AGPAT-1 and 2 were localized to the ER membrane consistent with their three transmembrane alpha helices and uncleaved signal sequence (31, 32). Intracellular localization of the remaining AGPAT proteins, has been determined using the PSORT program and suggests that AGPAT-3 and -4 are ER membrane integral proteins (33). The

regulation of individual AGPAT genes is uncharacterized.

1.2.3 *Phosphatidate Phosphohydrolase* (EC 3.1.3.4)

Two forms of PAPH activity exist. Several cDNAs for the PAPH-2 family or lipid phosphate phosphohydrolases have been cloned and these enzymes appear to play a role in signal transduction pathways by generating the lipid second messenger, DG (34). PAPH-1 activity exists in both cytosol and microsomal fractions. It has been proposed that long-chain fatty acids stimulate the translocation of PAPH-1 from the cytosol to the ER membrane where PA is hydrolyzed PA to form DG (35, 36). A cDNA encoding an enzyme with PAPH-1 activity has not been cloned, but this activity is considered the rate limiting step for *de novo* TG synthesis. There is a strong correlation between the activity of membrane associated PAPH-1 and the rate of TG synthesis (37). PAPH-1 activity is subject to dietary and hormone-induced changes (37). PAPH-1 activity was increased substantially in obese Zucker rats (22). PAPH-1 activity was increased 2-3 fold by glucagon, cAMP and dexamethasone in primary rat hepatocytes (38-40). The peak PAPH-1 activity occurred 2h after the peak plasma corticosterone concentration (41). It was hypothesized that enhanced PAPH-1 activity within the physiological range of corticosteroid levels enables the liver to increase its capacity to synthesize TG during the period of maximum feeding.

1.2.4 *Diacylglycerol acyltransferase* (EC 2.3.1.20)

DGAT catalyzes the final step in TG synthesis. Two DGAT cDNAs have been cloned. DGAT1, a homologue of ACAT, is

ubiquitously expressed in human and murine tissues with the highest levels of expression in the small intestine, colon, testis, thymus, heart and skeletal muscle (42). The mammalian DGAT2 cDNA is unrelated to the mammalian DGAT1 cDNA and was cloned based on its homology with a fungal DGAT cDNA (43, 44). Several additional murine and human DGAT2 related sequences exist but have not been characterized (44). DGAT2 is highly expressed in liver and adipose tissues that have a high capacity to synthesize TG (44). Analysis of the amino acid sequence that DGAT2 cDNA encodes, predicted a single transmembrane segment and a domain with homology to the glycerol binding domain of GPAT and AGPAT (33, 44). Further analysis of the amino acid sequence of DGAT2 by PSORT predicted the glycerol binding domain to be within the ER lumen where the synthesis of TG would be ideally linked to the site of VLDL assembly. A putative diacylglycerol binding motif of DGAT1, that is similar to the motif in protein kinase C, is predicted to be facing the cytosolic side of the ER (45). This would be consistent with the finding that two separate (overt and latent) DGAT activities exist in rat liver microsomes (46, 47). One may hypothesize that the overt DGAT activity preferentially synthesizes TG directed towards lipid storage within the cytosol whereas the latent DGAT activity synthesizes TG that is preferentially directed towards the ER lumen where it is assembled into lipoproteins (see 1.6.4). This possibility remains to be tested utilizing DGAT isoform specific antibodies and mice where the expression of individual DGAT genes have been over expressed or eliminated.

The relative contribution of each DGAT activity and the expression of the two DGAT genes was examined during postnatal

development in the rat liver, when enormous changes in dietary and hormonal conditions cause rapid changes in TG synthesis and secretion. It was found that the overt DGAT activity rose transiently up to day 4 postnatal and declined until weaning when it increased until it reached adult values (48). Latent DGAT activity increased continuously during suckling, then declined upon weaning onto a chow diet, though not a high fat diet (48). Interestingly, neither hepatic DGAT1 nor DGAT2 mRNA levels correlated with the postnatal changes observed in DGAT activity (48). In 3T3-L1 cells, it was demonstrated that DGAT1 was not regulated by phosphorylation/dephosphorylation mechanisms, but it appeared that translational efficiency regulated the level of DGAT1 protein within the cell (49).

The precise contribution of DGAT1 to TG synthesis was examined in mice that had the DGAT1 gene eliminated. The targeted deletion of the DGAT1 gene failed to eliminate the synthesis of TG in many tissues and the production of plasma TG was normal (50, 51). *Dgat1*$^{-/-}$ mice had dry fur that did not repel water, and female mice had a defect in their ability to lactate (50). In addition, DGAT1 null mice had a 50% reduction in fat pad content and were resistant to weight gain when fed a high fat diet that was associated with a 15% increase in daily total energy expenditure (50). DGAT1 null mice had increased sensitivity to insulin and leptin (51). When *Dgat1*$^{-/-}$ mice were crossed into the background of the obese leptin deficient *ob/ob* mouse, DGAT1 deficiency did not affect energy and glucose metabolism (51). Likewise, the DGAT1 null mouse had normal fur when crossed into the background of the *ob/ob* mouse and leptin injections caused the fur to become dry

(52). In addition, DGAT2 expression was increased in *ob/ob* mice, suggesting that the leptin pathway directly down regulates DGAT2 expression and in the absence of leptin, DGAT2 sufficiently compensated for the loss of DGAT1 mediated TG synthesis (51, 52). Indeed, others have observed a 3-fold increased overt and latent DGAT activities in liver microsomes from obese *ob/ob* mice relative to lean controls (53). DGAT1 was not necessary for intestinal TG absorption and chylomicron synthesis, although a high fat diet caused accumulation of cytosolic TG within the enterocytes of *Dgat1*$^{-/-}$ mice (54). Together, these results suggested that DGAT2 (or possibly diacylglycerol transacylase) activities sufficiently compensated for the targeted deletion of the DGAT1 gene in many tissues. Therefore, it is not surprising that more than one DGAT gene would exist as a backup mechanism for TG synthesis.

1.2.5 *Diacylglycerol transacylase* (EC 2.3.1.20)

Acyl-CoA independent mechanisms for TG formation are present in animal tissues. The isolation of a diacylglycerol transacylase (DGTA) from enteric microsomes demonstrated that the transacylation between two 1,2-DG molecules to form TG and MG occurs (55). The relative contribution of this pathway to TG synthesis is unknown since enzymes of the monoacylglycerol and phosphatidic acid pathways exist in the same sub-cellular compartment. DG molecules could be derived from either pathway. DGTA activity is low when compared to total DGAT activity (55, 56). However the presence of DGTA may be important for the synthesis of TG within the ER lumen since transport of acyl-CoA across the ER membrane would not be required for TG synthesis (47).

1.3 MONOACYLGLYCEROL PATHWAY

Within the intestinal lumen, lipases hydrolyze TG to free fatty acids and 2-MG. Since 2-MG is resistant to hydrolysis by pancreatic lipase, MG is taken up by enterocytes and undergoes resynthesis to TG. This process (Fig. 1.1) is initially catalyzed by monoacylglycerol acyltransferase (MGAT) to synthesize DG which then serves as a substrate for DGAT (see 1.2.4) or possibly DGTA (see 1.2.5). Most of the TG generated by the intestine is secreted as chylomicron particles.

1.3.1 *Monoacylglycerol acyltransferase* (EC 2.3.1.22)

MGAT activity in the small intestine is primarily responsible for the acylation of dietary MGs. MGAT activity is low in adult livers, but is high during the neonatal period and may prevent excessive breakdown of essential fatty acids (57, 58). In adult livers, MGAT activity is high in diabetic animals, but was low in obese Zucker rats (21, 59). Significant levels of MGAT activity are detected in white adipose tissue and kidney (21, 57). The different chromatographic and inhibition profiles of the rat intestinal and hepatic MGAT activities suggested that MGAT activity represents tissue specific isoenzymes (60, 61). A murine cDNA that is closely related to DGAT2 was identified as encoding a protein that possessed MGAT activity when expressed in insect cells (62). This gene, designated as MGAT1, was expressed in stomach, kidney, liver and adipose tissues of the mouse (62). Interestingly, MGAT1 was not expressed in the small intestine suggesting the existence of an additional MGAT gene.

1.4 NEUTRAL LIPID STORAGE

The ability to store neutral lipids plays a critical role in the ability of an organism to withstand fuel deprivation, and dysregulation of TG synthesis and excessive lipid storage contributes to the development of diseases such as obesity and diabetes. It appears that most cells and tissues have the ability to synthesize and store neutral lipids as lipid droplets within the cytosol (63). However, only a few tissues have the ability to store large quantities of neutral lipids. The pool of stored lipid serves a specialized function for the type of tissue. Heart and muscle store small quantities of TG as a reservoir of fatty acids for oxidation in the working muscle (64). Excessive TG storage within muscle and pancreatic beta-cells correlates with insulin resistance (65, 66). Low density lipoprotein (LDL) uptake and accumulation of neutral lipids by macrophages at the site of vascular injury is well characterised and known to promote the development of atheromas (67). Steroidogenic tissues such as the adrenal glands, testis and ovaries form small cholesterol ester rich lipid droplets of 0.5-1.5 μm diameter that provide a pool of steroid substrate for the synthesis of hormones (63) (see 1.7). The liver has adapted so that it stores TG in advance of its repackaging of the lipid as lipoprotein particles thereby regulating the rate of assembly and secretion in concert with the energy needs of the body (68) (see 1.6). For example, in the post-absorptive state, hepatic VLDL-TG secretion exceeds the rate of hepatic plasma non-esterfied fatty acid esterification, thereby acting as a buffer to store fatty acids taken up by the circulation as TG and releasing them at a later time as VLDL (69). White adipose tissue is quantitatively the most important tissue for the storage of

TG, forming cytosolic neutral lipid droplets that may exceed 50 μm in diameter, though the size varies based upon the nutritional status of the animal (see 1.5). Lipid storage within white adipose tissue serves as a reservoir for excess energy, as a heat insulator and now is recognized as an endocrine organ important to the regulation of energy intake through the secretion of hormones such as leptin (see 1.4.3) among others (70).

1.4.1 *Content and formation of intracellular lipid droplets*

Intracellular lipid droplets may be defined as a spherical particle composed of a core of neutral lipids such as TG and cholesteryl esters (CE) that are surrounded by a monolayer of amphipathic phospholipid and sterols. In addition, these lipid droplets are surrounded by a specific population of proteins that may be involved in lipid droplet synthesis, trafficking, stabilization and lipolysis, such as perilipin, adipophilin (also known as ADRP) and Tip47 (71). In addition, several ER associated proteins have been identified to be associated with lipid droplets (72, 73), though this may be an artifact of isolation it may also be due to co-isolation of ER lumenal lipid droplets with cytosolic lipid droplets. Alternatively, it may be that nascent lipid droplets have ER proteins associated with their surface when they are released into the cytosol from their site of synthesis, the ER. In addition it does not preclude the likelihood that ER membranes may transiently associate with and contact the lipid droplet within the cell (74, 75).

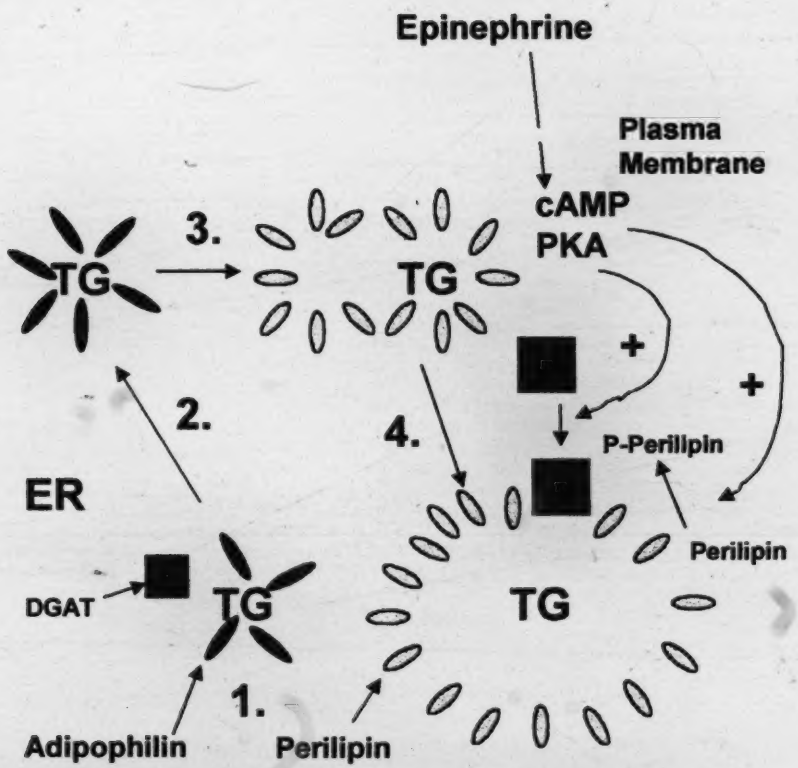


Figure 1.2- Mechanism for the formation of intracellular TG storage droplets in adipocytes. **1.** Nascent lipid droplets are synthesized at the ER surface. **2.** Droplets bud into the cytosol and tend to associate with the adipophilin protein. **3.** At later stages of differentiation, perilipin displaces adipophilin and lipid droplets undergo a regulated series of fusions to form large intracellular droplets. **4.** Perilipin coated lipid droplets undergo lipolysis when triggered by hormonally induced phosphorylation, by protein kinase A, of perilipin and cytosolic HSL.

Much of our understanding of the synthesis of neutral lipid droplets has been gained from studies utilizing the murine 3T3-L1 cell line. These cells can be stimulated to undergo differentiation from fibroblast-like precursor cells into terminally differentiated adipocytes through a mechanism that involves the induction of several adipocyte transcription factors, including the peroxisome proliferator-activated receptory (PPAR, see 1.4.2), followed by lipogenic gene expression and finally massive TG synthesis and storage within lipid droplets (76). TG is synthesized at the ER membrane and since large quantities of TG cannot be accommodated within the bilayer, spherical micro domains of TG are formed that represent precursors for the lipid droplet (63). Initially small lipid droplets are transferred into the cytosol (Fig. 1.2). The factors that regulate the transfer of TG out of the ER membrane into the aqueous environment remain obscure. These may involve proteins that associate with the surface of the lipid droplet, such as adipophilin, or it may involve proteins that interact with the cytoskeleton (77-79). As these cells mature into adipocytes, small cytosolic lipid droplets coalesce to form a few large lipid droplets that expand to occupy nearly the entire cell volume in the terminally differentiated adipocyte (63). In addition the protein composition of the lipid droplet changes during differentiation. In 3T3-L1 cells adipophilin is localized on the small lipid droplets during the early stages of lipid droplet formation (80). During the later process of adipocyte maturation, adipophilin is displaced from the lipid droplet surface and replaced by perilipin (80). While this result suggested that perilipin may be required for the fusion of small lipid droplets, ectopic expression of perilipin in 3T3-L1 fibroblasts did not promote

fusion of small lipid droplets on its own (81). In addition, perilipin replaces adipophilin on the surface of lipid droplets in steroidogenic cells in a similar fashion as in adipocytes, but fusion of the lipid droplets was not observed in these cells (82). Alternatively, perilipin is considered important for regulating the hormone stimulated lipolysis of the large lipid droplet in mature adipocytes (see 1.5.1) and steroidogenic cells (see 1.7.1) while adipophilin is not.

1.4.2 Peroxisome proliferator-activated receptors as regulators of TG storage

The transcriptional control of the expression of target genes involved in lipid metabolism provides a mechanism to regulate level of TG storage. PPARs represent a sub-family of the nuclear hormone receptor super family and are involved regulating pathways involving fatty acid uptake, storage as well as oxidation. These receptors bind to a direct repeat spaced by one nucleotide, fulfilling the consensus sequence 5'-AGGNCA N GGTCA-'3. PPARs interact with the upstream extended core hexamer whereas retinoid X-receptor (RXR) occupies the downstream motif (83, 84). Three related PPAR isotypes have been identified in vertebrates and were named PPARα, PPARδ and PPARγ. PPARα exhibits the highest affinity for polyunsaturated fatty acid ligands, such as linolenic acid, though all three isoforms bind polyunsaturated fatty acid ligands (85). Eicosanoids are fatty acids derived from arachidonic acid. Several eicosanoids such as 15-dexoy-Δ12,14PGJ2, are natural ligands for PPARγ (86, 87) and leukotriene B4 for PPARα (88). In addition, several synthetic ligands for the

PPARs are potent activators. Fibrates are commonly used as activators of PPARα whereas thiazolinediones are used as activators of PPARγ (89).

In adult rodents, PPARα is expressed in all tissues that have been tested with highest levels in brown fat, liver, kidney, heart and dueodenum (90, 91). Therefore, PPARα expression correlates with tissues that have a high capacity for mitochondrial and peroxisomal β-oxidation. Administration of fibrates to rodents resulted in numerous hepatic alterations including increased expression of mitochondrial and peroxisomal fatty acid metabolizing genes, as well as increased oxidation of fatty acids, decreased circulating TG and reduced adipose tissue stores (89). The decreased circulating TG was a consequence of increased VLDL clearance from the circulation by increased LPL activity and reduced apoCIII expression (92, 93). ApoCIII inhibits binding of lipoproteins to the endothelial surface and lipolysis by LPL. PPARα null mice have lower levels of hepatic expression of mitochondria genes including, very long chain and long chain acyl-CoA dehydrogenase, long chain acyl-CoA synthetase and 3-ketoacyl-CoA thiolase, compared to wild-type mice (94, 95). These changes are accompanied by marked hepatic lipid accumulation and inability to increase ketone body production in response to fasting, suggesting that fatty acid oxidation is impaired (94-99). Therefore it is clear that PPARα is a critical mediator of lipid homeostasis.

PPARδ is ubiquitously expressed with highest levels in heart, lung, kidney, skeletal muscle, testis, and at lower levels in the liver (96). PPARγ has a restricted pattern of expression, with highest

levels in white and brown adipose tissues (100). Lower levels are detected in the small intestine, skeletal muscle, liver and heart (101, 102).

PPARγ is expressed in only trace amounts in the fibroblastic pre-adipocyte cell line 3T3-L1. However, during adipocyte differentiation, PPARγ expression precedes the expression of late markers of differentiation, including aP2 and C/EBPα (103, 104). Thiazolinedione PPARγ activators, promote the conversion of pre-adipocytes into adipocytes (100, 105, 106). The targeted deletion of the PPARγ gene is embryonic lethal as placental development is defective (107, 108). Chimeric mice derived from wild-type ES cells and cells with a homozygous deletion of PPARγ showed exclusion of PPARγ null cells from white adipose tissue but not other tissues (109). A role for PPARγ in the induction of genes involved in the accumulation of lipids by adipocytes has been demonstrated. These genes include, the adipocyte fatty acid-binding protein, aP2, fatty acid transpoprter genes, FAT and FATP, as well as acyl-CoA synthetase and lipoprotein lipase (LPL) (93, 101-103, 110). Thus, it is clear that PPARγ is a key regulator of adipogenesis.

1.4.3 *Leptin as a regulator of TG storage*

The ability to communicate energy status to pathways regulating energy intake and expenditure is mediated by circulating hormones. The basis for obesity in the *ob/ob* mouse was determined to be the 16kDa leptin protein, which is absent in the *ob/ob* mouse (111). Leptin is a hormone secreted by adipocytes that functions to regulate the size of lipid stores through multiple

mechanisms acting on metabolic rate (112-116), fuel selection (117) and food consumption (112, 118, 119). The *db/db* obese mouse is deficient in the leptin receptor (Ob-R) and is unresponsive to endogenous or exogenous leptin (120). Ob-R is widely expressed, but is most highly expressed in the hypothalamus which is central to regulating feeding behaviour (121, 122). Leptin signals the size of the energy stores to the brain and other peripheral tissues. In the absence of the leptin signal, the body does not sense that there are adequate fat stores and in turn the organism becomes hyperphagic and obese. Plasma leptin levels correlate with adipose tissue mass and decrease in humans and mice after weight loss (123). Administration of plasma leptin to wild-type mice results in dose dependent decreases in body weight (2, 124). In addition, leptin appears to decrease lipogenesis (125, 126) and stimulate lipolysis (126-129) via a unique mechanism that does not involve free fatty acid release (see Chapter 5).

1.5 STORAGE OF TRIACYLGLYCEROL WITHIN ADIPOCYTES

In order to derive stored energy in times of metabolic need, free fatty acids must be released from the stored TG within the adipocyte. It has been estimated that approximately 10% of stored TG within adipocytes undergoes turnover on a daily basis (130, 131). Two classes of TG lipolysis are recognized to occur within the adipocyte. The well described hormonally regulated lipolysis (see 1.5.1) and the relatively uncharacterized basal lipolysis (see 1.5.2).

1.5.1 *Hormone Stimulated Lipolysis*

The hormone sensitive lipase (HSL) is recognized to catalyze both the hydrolysis of CE, as well as that of TG to DG and DG to MG (132, 133). A second enzyme, monoacylglycerol lipase (MGL) catalyzes the final hydrolysis of MG to free glycerol and fatty acids (134). MGL has not been studied in detail, though it does not appear to be acutely regulated. On the other hand, HSL is rapidly activated by lipolytic hormones such as catecholamines and glucagon by a mechanism that involves a hormone/G-protein induced increase in cyclic adenosine monophosphate (cAMP) (135). The increased cAMP activates cAMP-dependent protein kinase (PKA) to phosphorylate HSL, resulting in increased lipolytic activity (135, 136). Phosphorylation of HSL not only increases the activity of the enzyme, but also caused HSL to translocate from the cytosol to the lipid droplet (137). In addition, dephospohorylation of HSL occurs in response to insulin resulting in a reduction in lipolytic activity (138). The mechanism of the lipolytic stimulation also involves several proteins at the lipid droplet surface. For example, lipotransin binds PKA phosphorylated-HSL and it has been

proposed to translocate HSL to the lipid droplet (139). The N-terminal domain of HSL binds to the adipocyte lipid binding protein (ALBP) (140, 141). It has been proposed that ALBP may act to increase the hydrolytic activity of HSL by sequestering fatty acid products of lipolysis. The lipid droplet surface protein, perilipin may act as a barrier in non-stimulated cells preventing HSL access to the droplet (142). Overexpression of perilipin in NIH-3T3 or 3T3-L1 fibroblasts promotes TG storage and decreases the rate of TG hydrolysis (81, 143). PKA phosphorylates perilipin and stimulates lipolysis whereas mutation of the PKA phosphorylated sites on perilipin abolished the ability of lipolytic hormones to stimulate lipolysis (143, 144). In addition, insulin inhibits lipolysis and perilipin is dephosphorylated in response to insulin (145). Together these results demonstrated that the phosphorylation state of perilipin influenced the ability of HSL to interact with the lipid droplet (145). Targeted deletion of the murine perilipin gene resulted in a lean mouse with higher rates of basal lipolysis and blunted hormone stimulated lipolysis (146, 147). A 100-fold over expression of HSL in 3T3-L1 cells prevented TG accumulation (148). Since over expression caused HSL to be distributed throughout the cell, this result was consistent with the experiments that suggested perilipin regulated lipolytic activity through regulating the ability of HSL to access the lipid droplet. Overexpression permitted HSL to have unregulated access to lipids throughout the cell, thereby rendering the regulated translocation of HSL irrelevant. Therefore mechanisms that change the quantity of HSL within the cell could also affect overall lipolysis. For example, levels of adipose tissue HSL protein and mRNA are increased by starvation, glucocorticoids

and streptozotocin-induced diabetes and these conditions lead to increased lipolytic activity and reduced TG storage (149-151). Chronically high insulin and glucose concentrations increased HSL protein 40% and adipocyte lipolysis 40-65% (152).

1.5.2 *Basal Adipocyte Lipolysis*

Previously, it was observed that differences in the rate of basal lipolysis among various fat depots in the rat paralleled the amount of HSL protein found in the respective fat depot (153). Therefore it was a surprise that HSL was not absolutely required for TG lipolysis in the adipose tissue from *Hsl*$^{-/-}$ mice. The targeted deletion of the HSL gene eliminated only CE hydrolysis and reduced the basal TG lipolysis 50% indicating that substantial residual TG lipase activity exists in adipocytes (12, 13). Furthermore, the hormonal stimulation of lipolysis was blunted both *in vitro* and *in vivo*. The body weight and cold tolerance of *Hsl*$^{-/-}$ mice was unchanged (12, 13). At the moment, the only additional intracellular neutral TG lipase activity identified within the adipocyte is a microsomal TG hydrolase (TGH) (8). Inhibition of TGH utilizing a TGH specific inhibitor, reduced basal free fatty acid release by 3T3-L1 adipocytes (11, 14) and *in vivo* in hamsters (11) by approximately 40%. Together these results indicated that TGH contributed a major proportion of adipocyte basal lipolysis.

1.6 INTRACELLULAR HEPATOCYTE LIPID DROPLETS

The liver has the ability to store neutral lipids within the cytosol or secrete them into the circulation as VLDL. On average, a normal adult human liver stores about 5 μmol TG/g of liver weight, though the liver has the capacity to store much larger quantities of TG (68). Hepatic intracellular lipid storage droplets are smaller than those observed within adipocytes and only have the adipophilin protein on their surface (63). Interestingly, the TG stored within the cytosol of the hepatocyte is an important source of lipid for the lipoprotein particles that are secreted from the cell (see 1.6.3). Lipoprotein particles, much like intracellular lipid droplets, have a hydrophobic core of TG and CE surrounded by phospholipids and some free cholesterol. The surface of the lipoprotein particle is surrounded by amphipathic proteins that are involved in the assembly and secretion of the particle from the cell, as well as uptake of the particle from the circulation. The major apoprotein component of VLDL and the sole apoprotein of LDL is a single apolipoprotein B (apoB) molecule per particle (154). Gene targeting experiments in mice revealed that apoB is the sole apoprotein required for the secretion of a VLDL particle (155). ApoB is coded for by a single gene, though it occurs in two forms due to mRNA editing by a deaminase that converts codon 2153 from CAA to a stop codon (156, 157). The larger form, termed apoB100, is the sole form of apoB secreted by the human liver and the shorter form, apoB48, is secreted in addition to apoB100 by murine and rat livers (155). Both forms are secreted by the intestine and have the ability to assemble VLDL.

The relationship between high levels of LDL and

atherosclerosis has been known for several decades (158). LDL is derived from very-low density lipoprotein (VLDL) in the circulatory system via a complex series of reactions involving lipases, and the transfer of lipids and apoproteins among lipoproteins. A relationship between high levels of apo B in plasma and the risk of cardiovascular disease has been identified (159). Reduction of coronary artery disease has been observed in men treated with lipid lowering drugs that also cause a decrease in plasma apo B (160). Thus, there appears to be a positive link among the secretion of apo B from the liver, the levels of apo B in plasma and atherosclerosis, the principal cause of heart attack and stroke in the Western world (161).

1.6.1 *Mechanism of VLDL Assembly and Secretion*

The liver primarily secretes lipoprotein particles of a very low density. Much of our understanding of the mechanism of VLDL assembly and secretion is derived from cultured cells, both primary hepatocytes as well as the hepatoma cell lines including the McArdle RH7777 and HepG2 cells and more recently has been extended to include genetically engineered mice.

It is generally accepted that the hepatic assembly of VLDL occurs in two distinct steps (Fig. 1.3). Initially, apoB is translated on ribosomes attached to the ER membrane. Studies using McArdle RH7777 cells and primary rat hepatocytes demonstrated that the newly translated apoB was not integrated into the microsomal membrane, but initially associated with the membrane within the ER lumen (162, 163). In the first step, apoB is partially lipidated with a small quantity of TG, phospholipids and cholesterol forming a dense

particle. Since apoB is a large hydrophobic protein, chaperone assisted active folding of apoB appeared to be necessary during its translation and translocation into the ER lumen, as evidenced by its association with several ER lumen resident chaperones (164, 165). The microsomal triglyceride transfer protein (MTP), that has the ability to transfer lipid between membranes, is necessary for the assembly of apoB containing particles, as judged by its identification as the defective abetalipoproteinemia gene (166). MTP is composed of a 97kDa subunit that is complexed with the ER-folding enzyme protein disulfide isomerase (PDI) (167, 168).

In hepatoma cells that require incubation with fatty acids in order to secrete lower density apoB particles, assembly of fully lipidated particles occurred in two distinct steps (162, 169, 170). The subsequent addition of the bulk of the neutral lipid to the small dense apoB particle results in a very low density apoB particle that is released into the ER lumen for secretion into the circulatory system. A non-apoB associated neutral lipid droplet that was identified by electron microscopy within the lumen of the smooth ER is thought to be the source of the bulk of the lipid for the apoB particle (171). The bulk lipidation of apoB has been reported to be complete before entering the Golgi apparatus of rat hepatocytes, whereas it is reported to occur within a post-ER compartment in McArdle RH7777 cells (172-174).

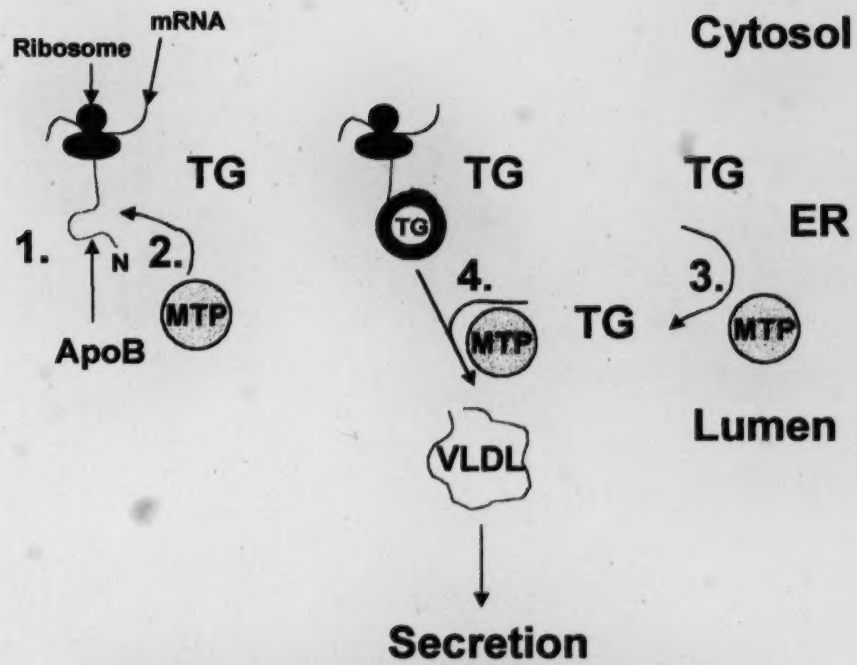


Figure 1.3- The hepatic assembly of very low density lipoproteins (VLDL) **1.** ApoB translation initiates on ribosomes attached to the ER membrane **2.** ApoB associates with a small quantity of lipids as it is co-translationally translocated into the ER lumen. **3.** TG is synthesized at the ER for a lipid droplet within the ER lumen. **4.** As more TG, phospholipid and cholesterol is transferred to the apoB particle, its size increases and a fully formed particle is released into the circulation.

1.6.2 *Regulation of VLDL secretion*

The transcription and translation of apoB is continuous so that newly synthesized apoB is always available for the assembly of lipids for secretion (175). A small quantity of the translated apoB undergoes assembly and secretion while a larger portion undergoes intracellular degradation (176-178). Lipid availability determines the percentage of apoB that is assembled into secretion-competent lipoprotein particles versus that which is misfolded and targeted for degradation (175, 177, 179). In hepatoma cells, active synthesis of lipids drives VLDL secretion since oleic acid promotes apoB secretion by hepatoma cells through increased TG synthesis and lipid availability (169, 180). Triacsin D blocked oleic acid stimulated apoB secretion through the inhibition TG synthesis without affecting cholesterol ester synthesis (181). It has been observed that CE synthesis has a regulatory role in VLDL synthesis (182, 183). However the targeted deletion of either the ACAT1 or ACAT2 genes in the mouse failed to demonstrate that CE synthesis was essential for lipoprotein production (184, 185). Phosphatidylcholine synthesis, a quantitatively small component of VLDL, is a potential regulator of VLDL assembly and secretion (186, 187).

The supply of free fatty acids to the liver is rate limiting for the secretion of VLDL-TG mice (188). Stearoyl-CoA desaturase (SCD) catalyzes the synthesis of monounsaturated fatty acids from saturated fatty acids. SCD-1 deficient mice have impaired TG and CE synthesis (189). Furthermore, these mice secrete low levels of VLDL-TG (189, 190). SCD-1 expression is tightly regulated by insulin and carbohydrates (191). Since high carbohydrate diets enhance the synthesis and secretion of TG by the liver and the

hepatic secretion of VLDL-TG by SCD-1 deficient mice do not respond to a high carbohydrate diet, suggests that SCD-1 can regulate VLDL secretion (190, 192).

The secretion of VLDL by the liver shows marked changes in response to nutritional and hormonal signals. Fasting decreases whereas carbohydrate feeding markedly increased the synthesis of all VLDL lipids (192). Levels of plasma hormones such as insulin, leptin and corticosteroids are directly related to feeding behavior and control the activity and expression of a number of lipid metabolic genes in the liver (193-195). In rodents, insulin is high in the fed state and low in the fasted state. Insulin inhibits hepatic VLDL secretion and promotes lipid synthesis (193). Therefore perturbations in levels of these hormones correlate with changes in plasma lipid levels.

MTP activity has the ability to regulate VLDL assembly. Inhibition of MTP lipid transfer blocked the translocation of apoB100 causing it to be co-translationally degraded at an early stage in the assembly process (196). Inhibition of the ability of MTP to bind apoB without affecting MTP lipid transfer activity was sufficient to inhibit apoB secretion from HepG2 cells (197). Since the association of MTP with PDI is required for its lipid transfer activity, and apoB interacts with MTP at the site where PDI binds MTP, it is likely that MTP acts as a chaperone to facilitate apoB folding independent of its lipid transfer activity (198, 199). In addition MTP may regulate the second step of VLDL assembly. The formation of the lipid droplet within the ER lumen is thought to be dependent upon MTP, though the bulk lipidation of the apoB particle may be MTP independent (162, 200-202).

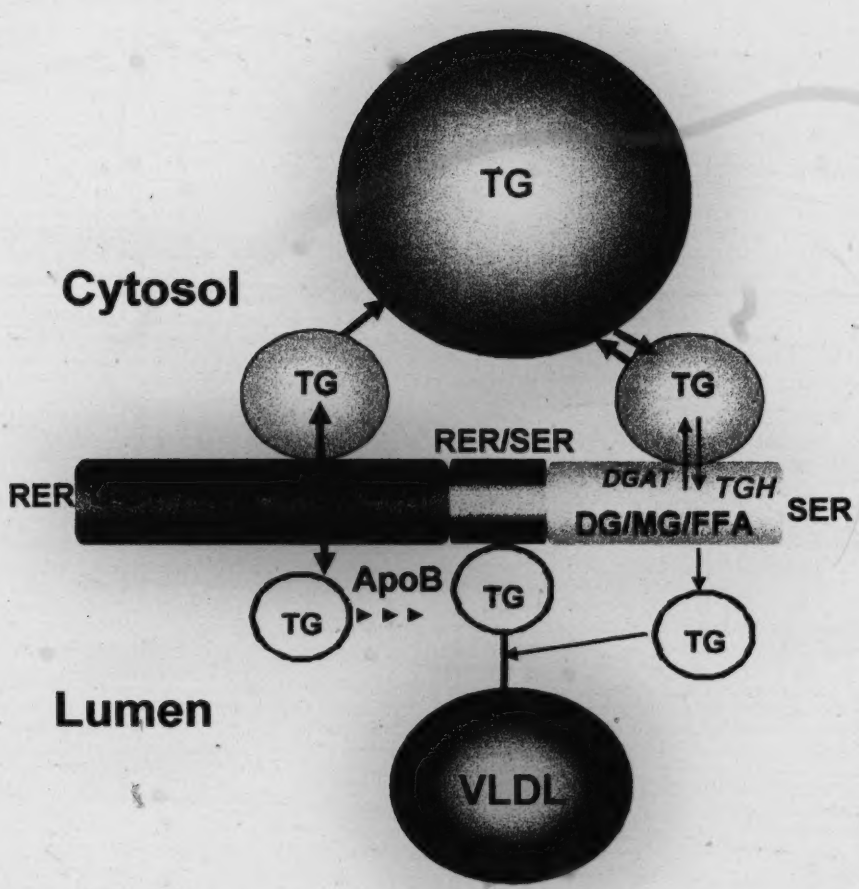


Fig. 1.4- The enzymology and topology of hepatic lipolysis and re-esterification. TG synthesized at the ER membrane may be directed towards secretion directly or towards storage within the cytosol. TG is mobilized from the cytosolic stores for secretion through the lipolytic action of a ER lumenal lipase such as TGH, triacylglycerol hydrolase. The lipolytic products are resynthesized within the ER lumen by DGAT, diacylglycerol acyltransferase.

1.6.3 *Mobilization of Stored TG from Hepatocytes*

Several groups, using different experimental approaches, have quantitatively determined that the TG storage droplet within hepatocytes undergoes a cycle of lipolysis followed by reesterification. Overall, the data is consistent with a proportion of the relased fatty acids being esterified to form TG that is secreted as VLDL, though the majority of the re-esterified TG was returned to the storage droplet.

Wiggins and Gibbons (203) pre-labeled the stored TG of cultured primary rat hepatocytes with ^{3}H-oleate and ^{14}C-glycerol and observed the distribution of the labeled TG over a subsequent 24h chase period in the absence of exogenously supplied fatty acids. These authors contend that any changes in the specific radioactivity of the two isotopes in TG requires uncoupling of ^{3}H-oleate and ^{14}C-glycerol which represents lipolysis followed by esterification. If the extent of decline in ^{14}C and ^{3}H specific radioactivity is of the same magnitiude, then the net lipolysis and esterification is zero, *de novo* synthesized TG can be supplied directly for secretion and the decline in the specific activity of cell and secreted TG would refelect the extent of *de novo* TG synthesis. Two significant observations were made by this study (203). 1) A 70% decline of the VLDL-TG ^{14}C label indicated that the original TG-glycerol pool was diluted with unlabeled glycerol and 2) The quantity of hydrolyzed TG that returned to the intracellular storage pool amounted to 1 pool per day, which was estimated to be 2-3 times greater than required to maintain TG secretion. Since the original TG-glycerol pool was diluted with unlabeled glycerol, lipolysis and re-esterification appeared to be essential for VLDL-TG assembly. Since the majority

of the TG that undergoes lipolysis and re-esterification was returned to the storage pool, it appeared that this step was not rate limiting.

Lankester *et al.* (204) used a dual labeling technique to differentiate between the incorporation of acyl chains into TG that were derived from exogenous and endogenous fatty acids. Further, these authors attempted to determine whether cytosolic TG is hydrolysed completely to fatty acid and glycerol or to just DG. The stored TG was pre-labeled with ^{3}H-oleate and then cultured a further 3h with ^{14}C-oleate. The authors observed exogenous ^{14}C fatty acids contributed only approximately 17% of total acyl chains secreted as TG, indicating that the majority of the acyl chains were derived from the pre-labeled cytosolic TG stores. Furthermore, the authors demonstrated that the cytosolic TG was not hydrolysed completely to fatty acid and glycerol. Since the ^{3}H fatty acids derived from cytosolic labeled TG was not available to the same extent as exogenous ^{14}C fatty acids for oxidation, ^{3}H labeled TG undergoes incomplete lipolysis to DG and is available for resynthesis as secreted TG.

Yang *et al.* (205) used chiral and reversed phase HPLC with mass spectrometry to reveal similarities in positional distribution and molecular association in the 1,2-DG acyl chains of secreted VLDL-TG and stored hepatic TG. However, the 2,3-DG acyl chains of secreted VLDL-TG were different from the stored hepatic TG. These authors calculated that 60% of secreted TG was derived via lipolysis to DG followed by esterification and 40% of secreted TG could have been derived from *de novo* TG synthesis. These authors added further evidence through the analyses of secreted TG following labeling of stored TG by ^{3}H-fatty acids or glycerol. The

authors calculated that 30-40% of the glycerol and fatty acids in VLDL-TG are not direct products of TG stored within the liver (206). Taken together these results were consistent with TG stored within the liver undergoing lipolysis to DG/MG and then undergoing re-esterification prior to their secretion.

1.6.4 *Enzymology and Topology of Hepatic TG Synthesis for VLDL Assembly*

The molecular mechanism and intracellular location of the enzymes responsible for the lipolysis and resynthesis of TG for VLDL secretion remain obscure. Since apoB containing lipoproteins are assembled within the ER lumen, it is likely that the synthesis of TG for VLDL assembly must be directed towards that compartment (Fig. 1.4). A lipase that mobilizes intracellular stored TG would likely be located within the ER in order to channel lipolytic products towards the ER for their resynthesis to TG at the site of VLDL assembly. Expression of the cytosolic lipase, HSL, in HepG2 cells directed fatty acids into the oxidative pathway as opposed to the secretory pathway (207). Furthermore, inhibition of lysosomal lipase by chloroquine did not affect the lipolysis of the intracellular stored TG (203). To that end, two candidate lipases localized to the microsomal lumen of the liver have been identified and termed a triacylglycerol hydrolase (TGH) and arylacetamide deacetylase (AADA) (68, 72). TGH is a lipase with a GXSXG catalytic serine motif and HXEL ER-retrieval sequence motif that localizes TGH to the ER lumen (6, 208). Immunocytochemical studies localize TGH expression exclusively to liver cells surrounding the capillary vessels, an area that is likely to be active in lipoprotein production

and secretion (72). Hepatoma cells that secrete poorly lipidated apoB particles are impaired in their ability to mobilize stored TG for secretion (209). Overexpression of TGH in McArdle RH7777 cells stimulated TG secretion through the increased utilization of TG stores for secretion (10). Transfection of HepG2 cells with AADA also stimulated TG secretion (68).

The transfer of fatty acids into the ER lumen may occur by several possible mechanisms. One possibility is that lipolysis occurs at sites where electron microscopy showed that the ER membrane is in contact with cytosolic lipid droplets (74, 75). At these contact points, lipolytic products would have increased solubility within the ER membrane and would be preferentially selected by the synthetic enzymes located within the ER membrane as opposed to being directed towards the oxidative pathway in the mitochondria (68). A second possibility is the transfer of lipolytically derived fatty acids as acylcarnitine derivatives. Microsomal carnitine acyltransferases have been demonstrated to exist (210, 211). Since tolbutamide inhibits microsomal carnitine acyltransferase and suppresses VLDL secretion by hepatocytes, it is likely that the microsomal carnitine acyltransferase system is an important component for the delivery of fatty acids to the ER lumen (203, 212).

The identity of the DGAT enzyme that synthesizes TG for VLDL assembly has not been established (see 1.2.4). However, it was established that a DGAT activity that synthesizes TG within the ER lumen exists (46-48). Different acyltransferases (or transacylases), acting in different subcellular compartments could be involved in the synthesis of TG and the extent of their respective

activities could channel TG preferentially for storage within a cytosolic lipid droplet or towards the secretory pathway (46, 204).

1.6.5 *Regulation of Hepatic TG Lipolysis and Re-esterification*

Since it has been demonstrated that intracellular TG lipolysis and re-esterification was necessary for the efficient transfer of TG stored within the cytosol to the sites of VLDL assembly, then altering the rate of hepatic TG lipolysis and reesterification could control the rate of VLDL secretion. When MTP was inhibited, the secretion of VLDL was decreased without a change in lipolysis (213). In this case, lipolytic products were recycled back to the cytosolic TG storage droplet. The hormonal and dietary factors known to regulate the rate of hepatic VLDL secretion could affect hepatic lipolysis and re-esterification (195). Insulin inhibits the secretion of TG by rat hepatocytes (214). However, insulin did not affect the dilution of total glycerol labeled TG, consistent with unchanged lipolysis and re-esterification, but merely increased the proportion of hydrolyzed TG that was recycled to the TG stores (203). Glucose on the other hand, increased hepatic TG secretion and increased the dilution of the glycerol label in pre-labeled TG, consistent with increased lipolysis and re-esterification (192, 215, 216). In addition, it was found that glucose phosphorylation was a necessary event for increased lipolysis and re-esterification since mannoheptulose, an inhibitor of glucose phosphorylation, abolished the stimulatory effect of glucose (216). Glucagon and cAMP accelerate intracellular lipolysis in hepatocytes, but direct fatty acids into the oxidative pathway as opposed to storage or secretion (217). It was found that glucagon did not affect dilution of the glycerol label in pre-labeled

TG, consistent with unchanged lipolysis and re-esterification (203).

1.7 ADRENAL GLAND LIPID STORAGE

The adrenal cortex accumulates numerous small lipid droplets that have a high CE content and are surrounded by perilipin (63). All steroid hormones are synthesized from free cholesterol. Since the adrenal cortex functions to synthesize a variety of different steroid hormones, mobilization of the stored CE provides the majority of the cholesterol for steroid hormone synthesis.

1.7.1 *Synthesis and Function of Glucocorticoids*

Glucocorticoids are one class of steroid hormones that are synthesized in the *zona fasciculata* section of the adrenal cortex. Stimulation of the hypothalamic-pituitary adrenal (HPA) axis causes the hypothalamus to release corticotropin releasing hormone (CRH) which then acts at the anterior pituitary to release adrenocorticotropic hormone (ACTH) which is the most potent stimulus of steroid hormone synthesis (218). ACTH acts at the adrenal cortex and binds to the plasma membrane ACTH G-protein coupled receptor of which in turn, stimulates the synthesis of cAMP and activates cholesterol esterase and mobilization of stored CE. Recently, two papers have demonstrated that HSL is the neutral cholesterol esterase in the adrenal gland and HSL was necessary for ACTH stimulated free cholesterol release from adrenal gland lipid droplets and for steroid hormone synthesis (219, 220). The release of free cholesterol, followed by its conversion to pregnenolone, cleavage of the cholesterol side chain, and a series of reactions in the ER and mitochondria lead to the production and

release of cortisol/corticosterone following ACTH stimulation (Fig. 1.5) (221, 222). The principal glucocorticoid in humans is cortisol whereas in rodents it is corticosterone (Fig. 1.6).

Once within the blood, glucocorticoids are transported to target organs where they elicit their metabolic effects to an extent determined by the number of glucocorticoid receptors in the cell of a particular tissue. Most tissues of animals contain measurable quantities of receptor making it theoretically possible for nearly all tissues of the body to respond to glucocorticoids. The liver is an important target organ because of the relatively large concentration of glucocorticoid receptors in the hepatocyte (about 65,000 per cell) (223). Once within the target cell, glucocorticoids may act by a variety of mechanisms to either activate or repress the expression of target genes through mechanisms that act at the transcriptional or post-transcriptional level (224-226).

1.7.2 *Role of Glucocorticoids in Energy Metabolism*

A daily rhythm of food intake in rodents with *ad lib* access to food is clearly apparent. Rodents are nocturnally active and feeding primarily occurs during the dark phase, accompanied by increased storage of glycogen and fat molecules (227-229). During the light phase, rodents eat little and primarily use energy mobilized from storage pools to support their metabolic needs. The secretion of glucocorticoids parallels changes in the food intake of rodents fed *ad lib*. In both humans and rats there is an anticipatory increase in ACTH and corticosteroid secretion that precedes the onset of the daily feeding cycle as well as discrete pulses of secretion that accompanies meals. Basal plasma corticosteroid levels peak

(about 20μg/dL) 2 hours before the peak of the diurnal feeding rhythm and are lowest (about 0.1μg/dL) 2 hours prior to the trough of food intake. This rhythm is driven by ACTH secretion (230). In rats, there is a feeding associated increase in plasma corticosterone (231, 232) that is also apparent in plasma cortisol in humans (233, 234). Thus, at the onset of a meal, there are acute widespread increases in autonomic and endocrine activity in rats and humans. With feeding, both corticosteroids and insulin are secreted. Increasing durations of fasting elevates plasma ACTH and glucocorticoids during the trough period in humans (235) and rodents (231, 236, 237). Fasting levels of plasma glucocorticoids usually do not exceed the peak concentration observed in the normal diurnal feeding rhythm. The major difference between fed and fasted conditions is that insulin secretion is high during feeding and low during fasting so that the synergistic effects of glucocorticoids in combination with insulin differ in the two conditions.

High circulating levels of glucocorticoids are observed in the human disease, Cushing's syndrome, which is associated with increased activity of the HPA axis causing increased food intake, fat cell growth, insulin resistance as well as decreased thermogenesis and energy expenditure (238-241). Reduction of the high circulating glucocorticoids prevents or reverses these changes (242, 243). Deficiency of circulating glucocorticoids (Addison's disease) is associated with weight loss.

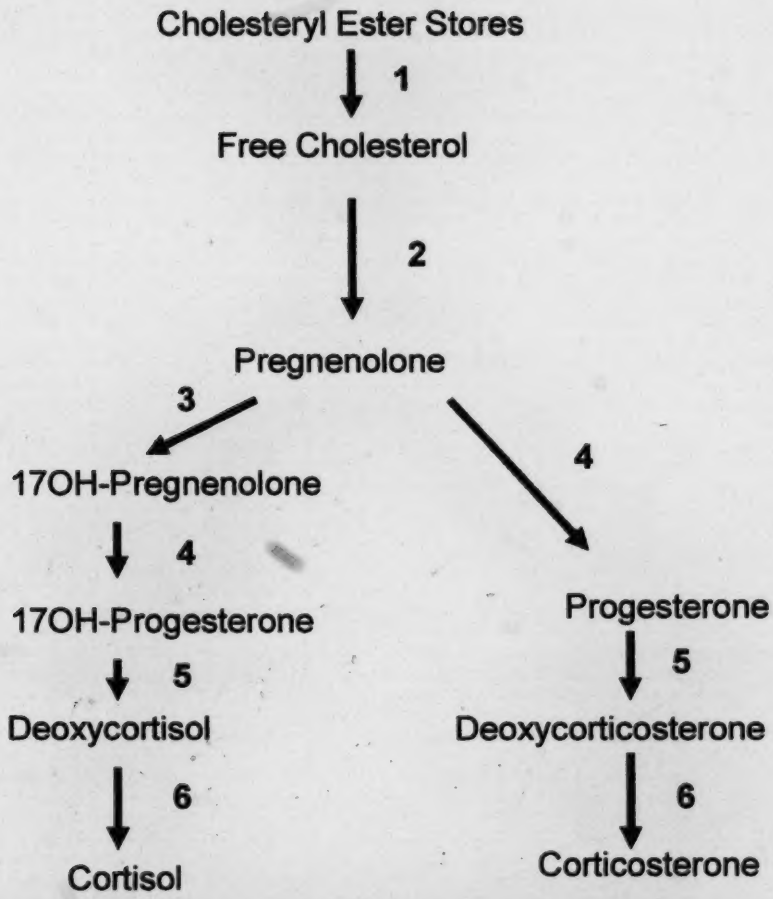
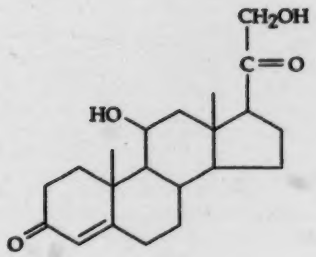
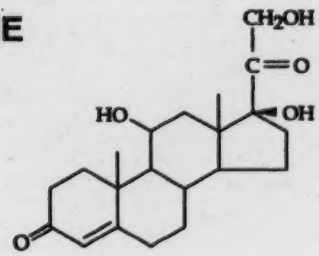


Figure 1.5- Pathways for the production of glucocorticoids by the adrenal gland. 1. Mobilization of free cholesterol from the storage pool by HSL, hormone sensitive lipase. 2. P450 side chain cleavage. 3. 17-α hydroxylation. 4. Oxidation of 3-β hydroxy group to an oxo group by 3-β steroid dehydrogenase isomerase. 5. 21-hydroxylation. 6. 11-β hydroxylation.

CORTISOL



CORTICOSTERONE



DEXAMETHASONE

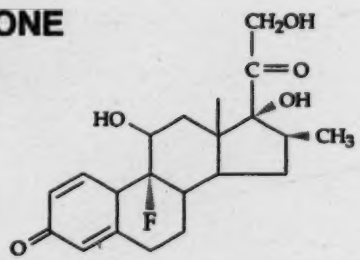


Fig. 1.6- The structures of the two major glucocorticoids cortisol and corticosterone that are secreted by the adrenal gland. Dexamethasone is a synthetic molecule that mimicks the physiological effects of natural glucocorticoids.

1.7.3 *Glucocorticoids and hepatic lipid synthesis, storage and secretion*

Glucocorticoids have dramatic effects on lipid synthesis. For example, excess glucocorticoids are associated with increased hepatic fatty acid and TG synthesis as well as increased circulating TG levels (244-249). In addition, diurnal variations of hepatic and adipose tissue lipogenesis essentially parallel that of food intake and plasma glucocorticoids (227, 228). Hepatic VLDL-TG secretion is also regulated by glucocorticoids and insulin and is highest during the post-absorptive period in rodents (193, 194, 250). Removal of glucocorticoids by adrenalectomy attenuated the development of obesity in a variety of genetic and dietary models of obesity (251, 252).

1.8 THESIS OBJECTIVE

In hepatocytes, stored TG is mobilized for VLDL assembly through lipolysis, followed by re-esterification. In order to establish a link between a microsomal TG hydrolase (TGH) and the provision of stored TG for VLDL secretion we will use several approaches. In Chapter 2, we have identified a novel murine cDNA, encoding the murine TGH protein and characterized its tissue expression profile. In Chapter 3, we have identified the murine TGH gene and its promoter, and examined its relation to carboxylesterases. Furthermore, we created a plasmid for the targeted deletion of the murine TGH gene in order to determine the role of TGH in the mobilization of stored TG in an intact animal. The potential for the regulation of VLDL assembly exists at a number of levels. The post-translational efficiency of apoB secretion depends upon the availability of lipids. Since TGH is proposed to have a role in the provision of lipids for VLDL assembly, potential for the regulated expression and activity of TGH to modulate the rate of VLDL secretion exists. We have chosen to investigate the regulation of TGH in several animal and cell culture models. In Chapter 4, the regulation of TGH by the glucocorticoid analog, dexamethasone is investigated since glucocorticoids have direct effects on hepatic lipid metabolism. In Chapter 5, the function of TGH in adipose tissue and the potential for the regulation of TGH through the PPAR and leptin pathways is investigated.

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CHAPTER 2

THE CLONING AND EXPRESSION OF A MURINE TRIACYLGLYCEROL HYDROLASE cDNA

The majority of this work was published in
Biochim. Biophys. Acta (2001) **1532**, 162-172.

2.1 Introduction

The liver assembles and secretes apolipoprotein B containing lipoproteins, mainly VLDL. The major lipid component of VLDL particles is TG. Studies from several laboratories indicate that lipid availability is the major determinant in the post-translational regulation of apo B secretion (1-4).

In the liver, TG is stored within cytosolic droplets of the hepatocyte (5). As described in Chapter 1, (see 1.6.3) various studies have demonstrated that the majority of the fatty acyl moieties of VLDL-TG are derived from this cytosolic TG storage pool and lipolysis of the stored TG to DG/MG followed by re-esterification to TG, is required to mobilize TG for VLDL secretion (6-8).

Triacylglycerol hydrolase (TGH) catalyzes the hydrolysis of long, medium and short chain TGs. TGH activity was purified from porcine liver microsomes (9). Nascent VLDL particles are assembled within the endoplasmic reticulum (ER). Localization of TGH to ER regions that are in close contact with cytosolic lipid droplets, suggested that TGH could participate in the lipolysis of stored TG destined for secretion as VLDL particles (10).

Recently, the cDNA encoding TGH was cloned from a rat liver cDNA library and it was demonstrated that TGH could mobilize intracellular TG stores (11). TGH is absent from hepatoma cells that secrete poorly lipidated apoB particles (10). When McArdle RH7777 hepatoma cells were transfected with the TGH cDNA, a two-fold increase, compared with vector transfected cells, in the rate of depletion of prelabelled TG stores was observed (11). TGH

expressing cells also secreted a 25% greater mass of TG into the medium and increased levels of apolipoprotein B in the VLDL range, compared to control cells. A lipase inhibitor, E600, decreased the secretion of TG and apoB by primary rat hepatocytes, but did not affect the secretion of TG by hepatoma cells that do not express TGH (12).

To examine the role of TGH in the mouse, a novel full-length murine cDNA encoding the murine TGH was cloned. Conserved amino acid sequences and structural features are discussed with regard to the lipolytic function of murine TGH. McArdle RH7777 hepatoma cells were transfected with the murine TGH cDNA and the expressed protein has *in vitro* esterase activity. Northern and Western blots demonstrate that TGH is expressed in a variety of mouse tissues that have the ability to store and secrete TG.

2.2 Materials and Methods

2.2.1 *Reagents*

Restriction endonucleases, modifying enzymes, random primer labeling kit, Dulbecco's modified Eagle's medium (DMEM), sodium pyruvate, penicillin/streptomycin, fetal bovine and horse serum and G-418 sulphate were obtained from Life Technologies Inc. Radioisotopes ([α-^{32}P]-dCTP, [γ-^{32}P]-dATP) were from Amersham Biotech. Western blotting reagents were obtained from Amersham Canada. TaKaRa Ex-Taq high fidelity polymerase was from Takara Biomedicals Ltd. The Topo-TA cloning kit was from Invitrogen. All other chemicals were purchased from Sigma.

2.2.2 *TGH-Specific PCR Primer Sequences*

All primers were synthesized at the DNA core facility, University of Alberta using a 394 DNA/RNA synthesizer (Applied Biosystems).

Ex2R: CAGGACTTTGCCTTTAACAGTGTTC; Ex5R: AAGTGACC CCAGTTTCCCCG; Ex10F: ATTCTCTCTTGTGGAAGTCCTACC; P-TGHII: GAGCAAAGTTGGCCCAGTATTTCATCACCATTTTGC TGAG; TGH3END: AAGATTGGTGCCTCAACTCAGG

2.2.3 *Identification of transcription start site*

CapSite™ cDNA (Nippon Gene) was a kind gift of Dr. Kozo Ishidate (Tokyo Dental and Medical University, Japan). Capped mRNA with intact 5' ends isolated from murine liver were used to identify the 5' end of the mRNA by reverse transcription-polymerase chain reaction, as described previously (13). PCR was performed

initially using the murine TGH-specific anti-sense primer, Ex5R, and a primer complementary to the attached linker, IRC. Nested PCR using the mouse TGH-specific anti-sense primer Ex2R and IIRC followed. Both amplifications were performed at 95°C 45 s., 58°C 45 s., 72°C 45 s. for 25 cycles. The resulting product was cloned into pCR2.1-Topo (Invitrogen), following the manufacturer's protocol. Recombinant plasmid DNA was sequenced as described previously to identify the transcriptional start site.

2.2.4 *Identification of the 3' untranslated sequences*

3'-untranslated sequences were determined using the 3'-RACE protocol, described previously (14). Briefly, murine liver total RNA was isolated, treated with RNase-free DNase I (Life Technologies) and reverse transcribed using an oligo-dT_{20} primer and Superscript II reverse transcriptase (Life Technologies) according to the manufacturer's instructions. PCR was performed on the reverse transcription products using an oligo dT anti-sense primer (Ad68-dT) containing an adapter sequence and a sense primer, Ex10F, followed by a second round of semi-nested PCR using the sense strand primer TGH3END. Both amplifications were performed at 95°C 1 min., 50°C 5 min., 72°C 20 min., 95°C 45 s., 57°C 1 min., 72°C 1 min. for 30 cycles. The resulting product was cloned into pCR2.1-Topo (Invitrogen), following the manufacturer's protocol. Cloned plasmid DNA was sequenced in both directions, as described previously, to identify the sequences preceding the poly(A) tail.

2.2.5 *RNA isolation and northern analysis*

Total murine tissue RNA was isolated using Trizol Reagent (Life Technologies) according to the manufacturer's instructions. To perform Northern blot analysis, total RNA was separated in a 1% agarose/2.2M formaldehyde gel and transferred to a Hybond-N+ nylon membrane (Amersham) using a vacuum blotter according to the manufacturer's instructions. Pre-hybridization and hybridization steps were carried out in 0.144 M $NaHPO_4$, 7% SDS, 2 μM $Na_4P_2O_7$, 2 μM EDTA, 100 μg ml^{-1} sheared salmon testes DNA (Sigma) and 1.5 x 10^6 cpm ml^{-1} ^{32}P-labelled 40bp oligonucleotide P-TGHII at 55 °C.

2.2.6 *Murine TGH cDNA expression*

A sequence containing the entire coding region of the murine TGH cDNA was excised from pCR2.1 plasmid by *Eco*RI digestion, and ligated into pCI-neo vector (Promega). Calcium phosphate mediated transfection of McArdle RH7777 cells was performed with 10μg of pCI-neo plasmid with or without the murine TGH cDNA and clones were selected for their resistance to the antibiotic G-418 (0.08% w/v). Transfected McArdle RH7777 cells were grown in DMEM supplemented with 10% horse serum and 10% foetal bovine serum, and penicillin/streptomycin (40 units/ml). Cells were maintained at 37°C in humidified air containing 5% CO_2.

2.2.7 *Lipase assay*

Lipolytic activity in cell lysates isolated from empty vector transfected and murine TGH cDNA-transfected McArdle RH7777 cells was assessed using the chromogenic substrate, *p*-nitrophenyl laurate (9).

2.2.8 *Immunoblot analysis*

McArdle RH7777 cell homogenates (35 μg of protein) were electrophoresed on an SDS/10% (w/v) polyacrylamide gel, transferred to a nitrocellulose membrane, and the expression of TGH was analysed by blotting with the anti-TGH polyclonal antibody as described previously (9).

2.2.9 *Database searches*

BLAST was used to search the Genbank and EMBL databases. PSI-Pred and Predict Protein servers were used to analyze the murine protein for secondary structure features. Swiss-PDB viewer version 3.7 was used to model the structure of the murine TGH protein based upon the X-ray crystal structure of the rabbit carboxylesterase, Protein Databank Accession Number, 1K4Y.

2.3 Results

2.3.1 *Cloning of the full length cDNA encoding the murine triacylglycerol hydrolase*

Previously, a λgt11 cDNA library constructed from murine liver was screened by PCR using primers complementary to the previously cloned rat TGH cDNA (11). Two positive clones, 1.7 kb in length, containing the entire coding region were isolated and sequenced. In order to obtain the full-length cDNA sequence of the murine TGH cDNA, a CapSite cDNA prepared from murine liver was amplified by PCR (13). A single 250 bp long PCR product was cloned that overlapped the previously cloned cDNA by 190 bp. Several clones were sequenced and each clone had a single cap-site located 60 bp upstream of the translation initiation site of the transcript. The 3' end of the mouse TGH cDNA was determined using a 3'RACE protocol (14). A 300 bp PCR product was generated. This product was cloned and sequenced in both directions. Several clones were sequenced to verify their identity as the 3'untranslated sequence of the murine TGH cDNA. These sequences overlapped the previously cloned murine TGH cDNA by approximately 100 bp and contained approximately 200 bp of additional sequence.

The full-length murine cDNA for TGH, Genbank Accession No. AF378751, and its deduced amino acid sequence are shown in Figure 2.1. The cDNA is 1962 bp long with an open reading frame of 1695 bp. The putative start codon is preceded by a 60bp long 5'-untranslated sequence. The putative stop codon is followed by a

187 bp long 3'-untranslated sequence that contains several poly-adenylation signals and is followed by a 20bp poly-adenylated tail. The size of the mouse TGH cDNA is consistent with the 2kb signal detected by Northern blot (see Fig. 2.5 and 2.6).

2.3.2 *Predicted amino acid sequence of the protein encoded by the murine TGH cDNA*

The open reading frame of the murine TGH cDNA encodes a protein of 565 amino acids. Several protein motifs in the murine TGH protein are apparent (Fig. 2.1). The deduced protein sequence has an N-terminal 18 amino acid signal peptide that would presumably direct the protein into the lumen of the ER where TGH has been immunodetected (10). The Ser 222, Glu 353 and His 466 residues are highly conserved and are predicted to form the TGH catalytic triad (15, 16). Two potential N-glycosylation sites were identified at Asn 79 and Asn 489. The sequence contains five Cys residues, although the mature protein contains four. The deduced protein sequence also contains a hydrophobic stretch of amino acids 414-429 that may be involved in lipid binding (17-23). A C-terminal HVEL sequence for the possible retention of TGH within the ER lumen is present (24).

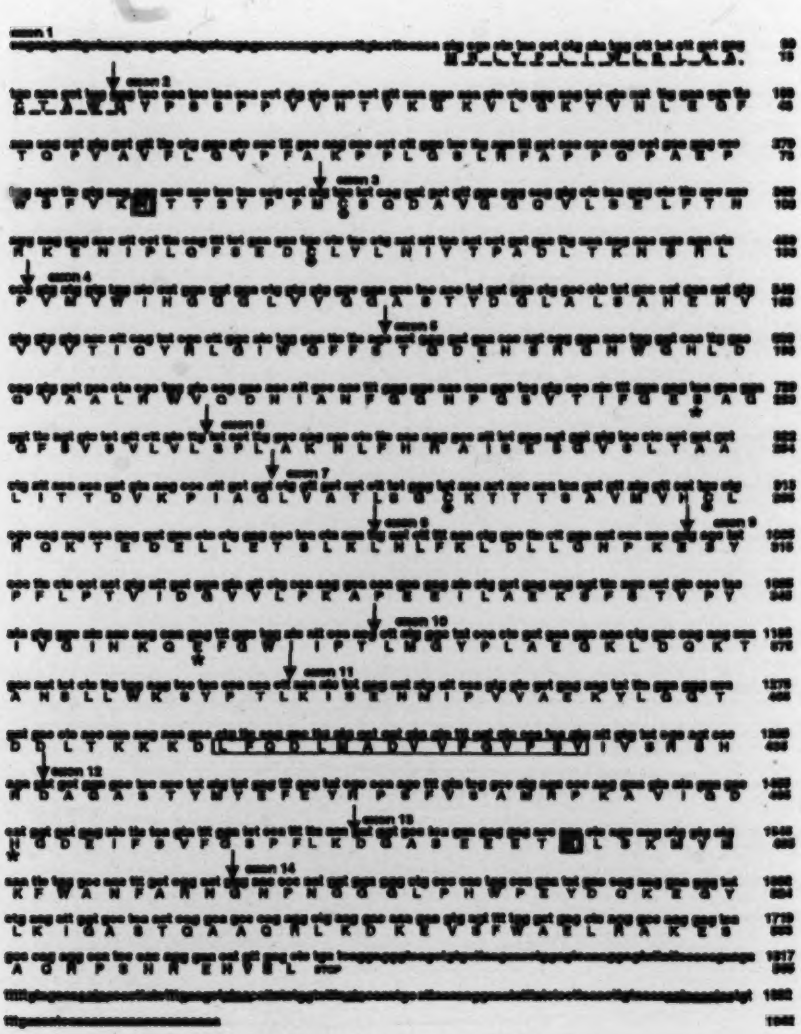


Figure 2.1- The murine TGHcDNA sequence and the deduced amino acid sequence of the corresponding protein. The first nucleotide of the cDNA and the first Met residue are numbered as 1. The signal peptide is indicated by the dashed line. The active site Ser, Glu, His residues are labeled with an asterix. A putative lipid binding domain is boxed. Putative N-linked glycosylation sites are boxed in bold. A C-terminal HVEL putative ER retrieval sequence is indicated by outlined letters. Poly-adenylation signals in the cDNA are underlined. The locations of intron-exon boundaries are indicated by an arrow.

2.3.3 *Murine triacylglycerol hydrolase is a member of the CES1A class of carboxylesterases*

BLAST searches of GenBank and EMBL databases were used to identify existing sequences with a high degree of identity to the murine TGH (Table 2.1). The amino acid sequence of TGH shows only minimal identity to previously identified lipases (16, 25), but more than 70% identity to carboxylesterases (15), suggesting their common evolutionary origin. Sequence comparisons revealed that the murine TGH protein clearly belongs to the CES1A class of carboxylesterases (15). An alignment of the murine TGH amino acid sequence to orthologous rat, rabbit, monkey and human carboxylesterases revealed that catalytic residues are conserved among a variety of mammalian species (Fig. 2.2).

Table 2.1- Comparison of the deduced amino acid sequence of mouse TGH and the sequences of previously identified esterases.

Name	Species	Tissue	Percent Identity	Ref.
Br3 carboxylesterase	human	brain	98	58
pI 6.1 esterase	rat	liver	93	27
carboxylesterase	rat	adipocyte	93	30
carboxylesterase	hamster	liver	89	59
carboxylesterase	monkey	liver	79	60
carboxylesterase	rabbit	liver	78	31
carboxylesterase	human	liver, macrophage	78	53, 61
Esterase-X	mouse	liver	76	62
Esterase-22	mouse	liver	76	63
Glycerol ester hydrolase	pig	liver	76	64
Esterase-4	rat	liver	72	34
Serum carboxylesterase	rat	liver	72	65
Es-N	mouse	liver	69	66
CE2	human	liver, intestine	46	67
Es-male	mouse	liver	40	68
acetylcholine esterase	mouse	liver	29	69
Carboxyl ester lipase	mouse	liver	28	70
AADA	mouse	liver	16	71
Hormone sensitive lipase	mouse	adipocyte	14	25

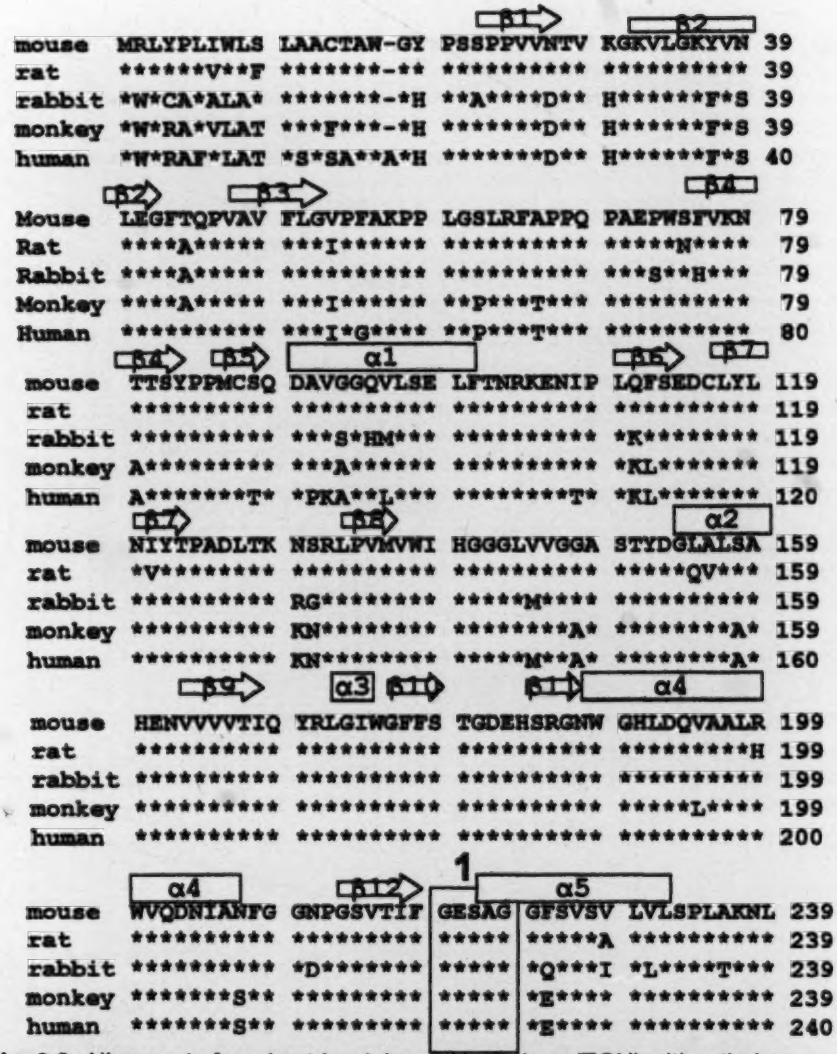
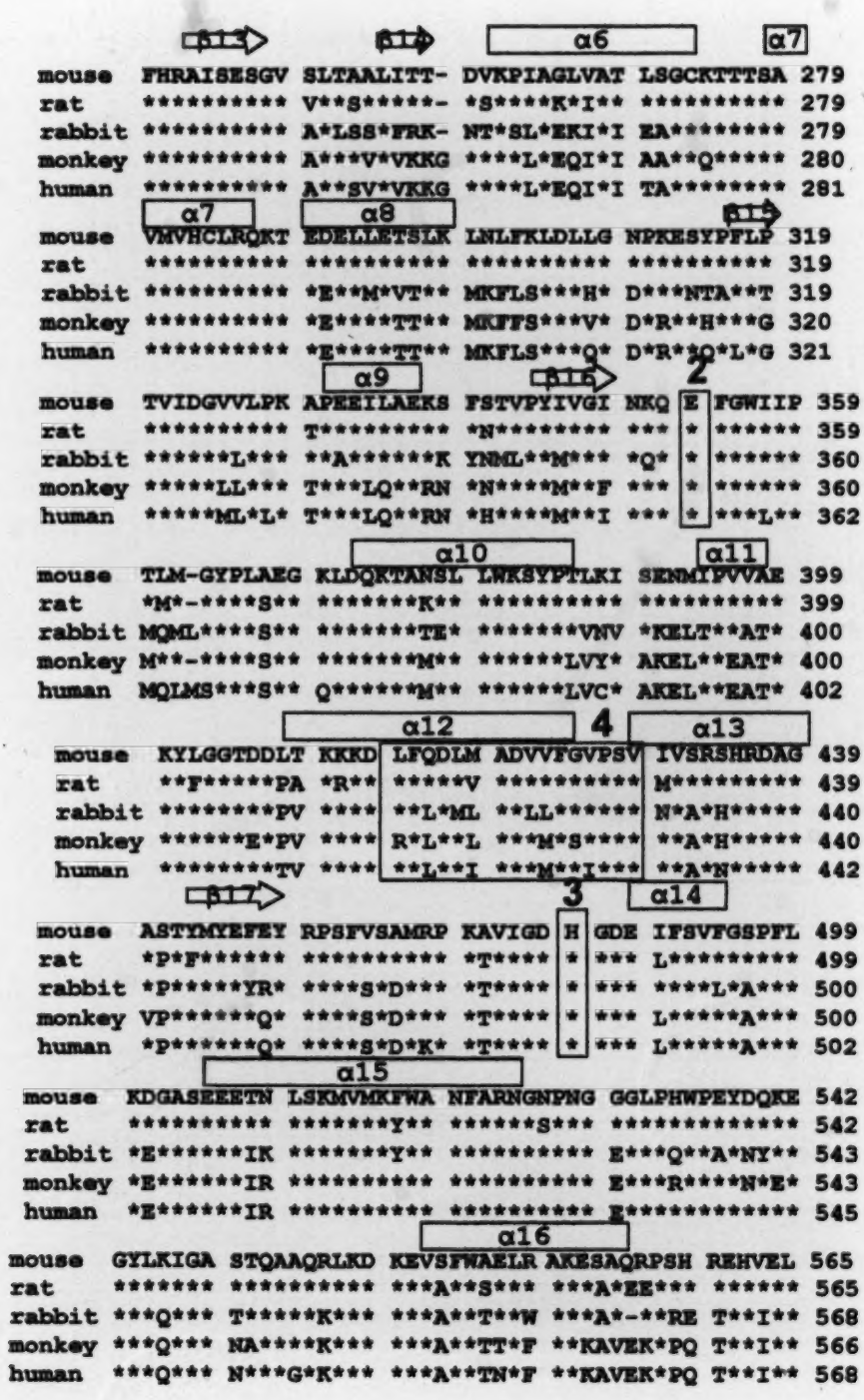

β1
β2
mouse MRLYPLIWLS LAACTAW-GY PSSPPVVNTV KGKVLGKYVN 39
rat ******V**F *******-** ********** ********** 39
rabbit *W*CA*ALA* *******-*H **A****D** H******F*S 39
monkey *W*RA*VLAT ***F***-*H *******D** H******F*S 39
human *W*RAF*LAT *S*SA**A*H *******D** H******F*S 40
β2
β3
β4
Mouse LEGFTQPVAV FLGVPFAKPP LGSLRFAPPQ PAEPWSFVKN 79
Rat ****A***** ***I****** ********** *****N**** 79
Rabbit ****A***** ********** ********** ***S**H*** 79
Monkey ****A***** ***I****** **P***T*** ********** 79
Human ********** ***I*G**** **P***T*** ********** 80
β4
β5
α1
β6
β7
mouse TTSYPPMCSQ DAVGGQVLSE LFTNRKENIP LQFSEDCLYL 119
rat ********** ********** ********** ********** 119
rabbit ********** ***S*HM*** ********** *K******** 119
monkey A********** ***A****** ********** *KL******* 119
human A********T* *PKA**L*** *********T* *KL******* 120
β7
β8
α2
mouse NIYTPADLTK NSRLPVMVWI HGGGLVVGGA STYDGLALSA 159
rat *V********* ********** ********** *****QV*** 159
rabbit ********** RG******** *****M**** ********** 159
monkey ********** KN******** ********A* ********A* 159
human ********** KN******** *****M**A* ********A* 160
β9
α3
β10
β11
α4
mouse HENVVVVTIQ YRLGIWGFFS TGDEHSRGNW GHLDQVAALR 199
rat ********** ********** ********** *********H 199
rabbit ********** ********** ********** ********** 199
monkey ********** ********** ********** *****L**** 199
human ********** ********** ********** ********** 200
1
α4
β12
α5
mouse WVQDNIANFG GNPGSVTIF GESAG GFSVSV LVLSPLAKNL 239
rat ********** ********* ***** *****A ********** 239
rabbit ********** *D******* ***** *Q***I *L****T*** 239
monkey *******S** ********* ***** *E***** ********** 239
human *******S** ********* ***** *E***** ********** 240

Fig. 2.2- Alignment of murine triacylglycerol hydrolase (TGH) with orthologous mammalian carboxylesterases. Murine TGH single letter coding sequence is shown at the top. The sequences for rat pI 6.1 esterase (27), rabbit liver carboxylesterase (31), a monkey liver carboxylesterase (60), and a human liver carboxylesterase (61) are aligned. Amino acid residues identical to murine TGH are represented by an asterix. Non-aligned residues are indicated by the single letter amino acid code. Thick yellow arrows above the sequence are regions forming beta sheets in the murine TGH protein. Blue rectangles indicate regions forming alpha helices in the murine TGH protein. **1**, GXSXG catalytic serine motif **2**, catalytic glutamic acid **3**, catalytic histidine **4**, putative neutral lipid binding sequence.


β13 β14 α6 α7
mouse FHRAISESGV SLTAALITT- DVKPIAGLVAT LSGCKTTTSA 279
rat ********** V**S*****- *S****K*I** ********** 279
rabbit ********** A*LSS*FRK- NT*SL*EKI*I EA********* 279
monkey ********** A***V*VKKG ****L*EQI*I AA**Q***** 280
human ********** A**SV*VKKG ****L*EQI*I TA********* 281
α7 α8 β15
mouse VMVHCLRQKT EDELLETSLK LNLFKLDLLG NPKESYPFLP 319
rat ********** ********** ********** ********** 319
rabbit ********** *E**M*VT** MKFLS***H* D***NTA**T 319
monkey ********** *E*****TT** MKFFS***V* D*R**H***G 320
human ********** *E*****TT** MKFLS***Q* D*R**Q*L*G 321
α9 β16 2
mouse TVIDGVVLPK APEEILAEKS FSTVPYIVGI NKQ E FGWIIP 359
rat ********** T********* *N******** *** * ****** 359
rabbit ******L*** **A******K YNML**M*** *Q* * ****** 360
monkey *****LL*** T***LQ**RN *N****M**F *** * ****** 360
human *****ML*L* T***LQ**RN *H****M**I *** * ***L** 362
α10 α11
mouse TLM-GYPLAEG KLDQKTANSL LWKSYPTLKI SENMIPVVAE 399
rat *M*-****S** *******K** ********** ********** 399
rabbit MQML****S** *******TE* *******VNV *KELT**AT* 400
monkey M**-****S** *******M** ******LVY* AKEL**EAT* 400
human MQLMS***S** Q******M** ******LVC* AKEL**EAT* 402
α12 4 α13
mouse KYLGGTDDLT KKKD LFQDLM ADVVFGVPSV IVSRSHRDAG 439
rat **F******PA *R** *****V ********** M********** 439
rabbit ********PV **** **L*ML **LL****** N*A*H***** 440
monkey ******E*PV **** R*L**L ***M*S**** **A*H***** 440
human ********TV **** **L**I ***M**I*** **A*N***** 442
β17 3 α14
mouse ASTYMYEFEY RPSFVSAMRP KAVIGD H GDE IFSVFGSPFL 499
rat *P*F****** ********** *T**** * *** L********* 499
rabbit *P******YR* ****S*D*** *T**** * *** ****L*A*** 500
monkey VP******Q* ****S*D*** *T**** * *** L*****A*** 500
human *P******Q* ****S*D*K* *T**** * *** L*****A*** 502
α15
mouse KDGASEEETN LSKMVMKFWA NFARNGNPNG GGLPHWPEYDQKE 542
rat ********** *******Y** ******S*** ********** 542
rabbit *E*******IK *******Y** ********** E***Q**A*NY** 543
monkey *E*******IR ********** ********** E***R****N*E* 543
human *E*******IR ********** ********** E********** 545
α16
mouse GYLKIGA STQAAQRLKD KEVSFWAELR AKESAQRPSH REHVEL 565
rat ******* ********** ***A**S*** ***A*EE*** ****** 565
rabbit ***Q*** T******K*** ***A**T**W ***A*-**RE T**I** 568
monkey ***Q*** NA*****K*** ***A**TT*F **KAVEK*PQ T**I** 566
human ***Q*** N***G*K*** ***A**TN*F **KAVEK*PQ T**I** 568

2.3.4 *Identification of secondary structure elements, overall structure and catalytic domains of the murine triacylglycerol hydrolase*

Analysis of the murine TGH protein sequence with PSI-Pred and Predict Protein secondary structure prediction programs rendered a succession of strong and well defined predictions for 16 alpha-helices and 17 beta-strands throughout the amino acid sequence (Fig. 2.2). In addition, these elements are strongly conserved among orthologus carboxylesterases from rat, rabbit, monkey and human. Therefore, the strong alignment of secondary structure elements and conserved residues allow the building of a three-dimensional model (Fig. 2.3), based on the crystal structure of the rabbit carboxylesterase and shows the overall topology of the protein encoded by the murine TGH cDNA (26). The catalytic Ser222 is located at the bottom of a deep active site cleft approximately in the center of the molecule which would offer a hydrophobic environment for a lipid substrate. The Glu353 and His466 residues that are proposed to participate in the catalytic triad are located adjacent to the two disordered loops within the active site cleft. The substrate binding region of TGH is formed by upper and lower jaws that surround the active site cleft similar to that observed for other lipases. The upper jaw is composed of four alpha helices 10-13, that include the putative lipid binding domain. The lower jaw is composed of two alpha helices 1 and 8 and the loop between beta strand 15 and alpha helix 8.

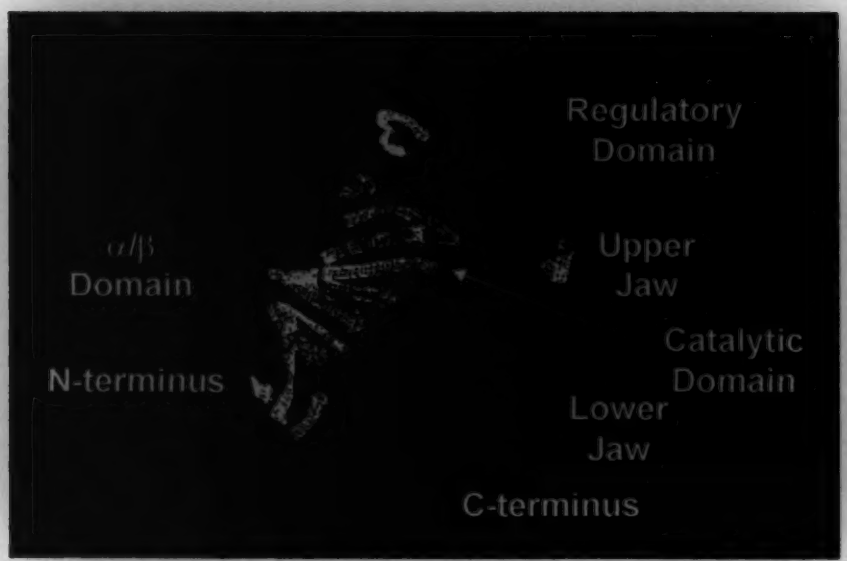


Figure 2.3- Modeled structure of the murine TGH protein. The structure of the murine TGH protein was modeled based upon the X-ray crystal structure of a rabbit carboxylesterase (26). Alpha helices are green. Beta-strands are yellow. Random coils are black. Catalytic residues are indicated in red. The alpha helix forming the lipid binding domain is indicated in blue.

2.3.5 *The murine triacylglycerol hydrolase cDNA encodes a functional protein with carboxylesterase activity*

The observation of active site residues and secondary and tertiary elements that were highly conserved among the carboxylesterases suggested that the cloned murine TGH cDNA encoded a functional protein. Previously it was observed that rat McArdle RH7777 hepatoma cells do not express the TGH protein (10). To demonstrate that the murine TGH is indeed functional, we stably transfected the murine TGH cDNA into rat McArdle RH7777 hepatoma cells. The transfected cells exhibited a 3-fold increase in the hydrolysis of *p*-nitrophenyl-laurate over empty-vector transfected cells (Fig. 2.4A). An immuno-detectable band migrating at 60 kDa on SDS-PAGE gels at the same position as TGH from mouse liver microsomes was present in lysates derived from murine TGH cDNA transfected cells (Fig. 2.4B). Therefore, the murine TGH cDNA encodes a functional protein that has carboxylesterase activity.

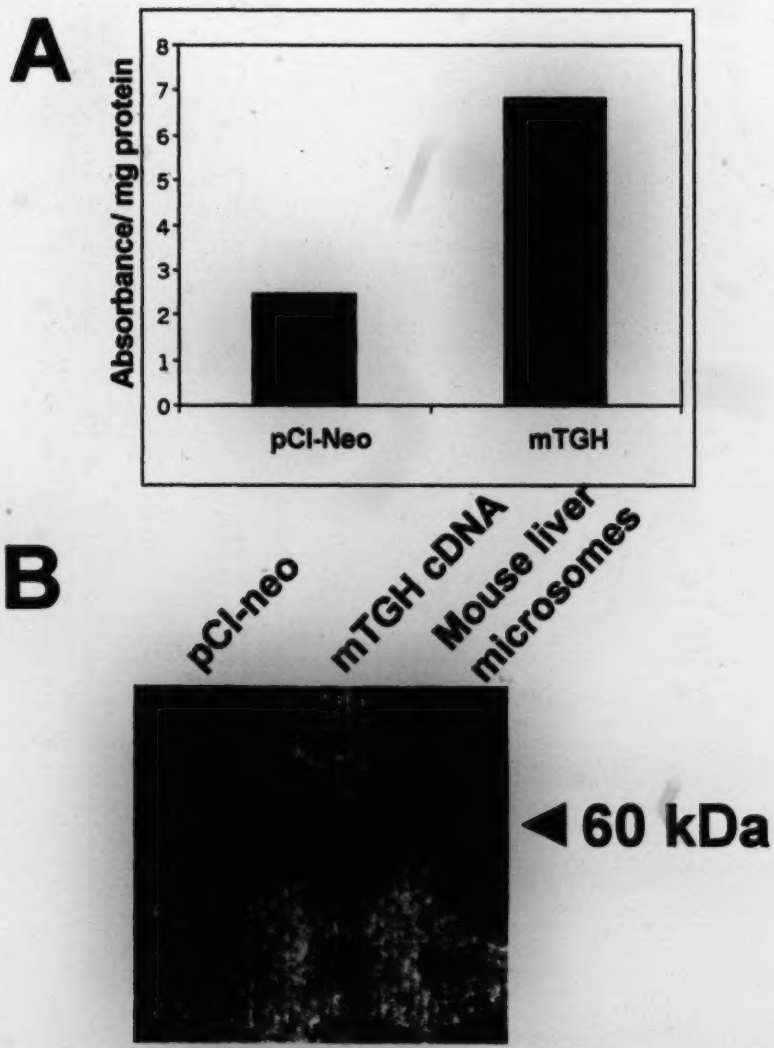


Figure 2.4: Expression of the murine TGH cDNA in McArdleRH7777 cells.

A. Lysates from cells stably transfected with the murine TGH cDNA in the pCI-neo vector were prepared and activity towards *p*-nitrophenyl laurate was measured.

B. Western blot of 30μg cell lysate from vector transfected and murine TGH transfected cells and 10 μg of mouse liver microsomes was performed. Similar results were obtained from other murine TGH cDNA-transfected McArdle cells.

2.3.6 *Murine triacylglycerol hydrolase is highly expressed in liver, but also in heart, kidney, adipose and intestine.*

Carboxylesterase activities have been reported in a wide variety of mammalian tissues (15). Since all carboxylesterases have the ability to catalyze the hydrolysis of ester bonds, measuring carboxylesterase activity in individual tissues does not accurately distinguish among carboxylesterase isoforms. Therefore, we endeavored to use a combination of Western and Northern blotting using TGH specific probes to detect the TGH protein and mRNA in various tissues (Figs. 2.5 and 2.6). Northern blot analysis revealed a single band of approximately 2kb that agrees with the size of the full length murine TGH cDNA (Fig. 2.1). Western blot analysis revealed a band of approximately 60kDa. Murine TGH proteins migrated on SDS gels at a slightly higher molecular weight in kidney, adipose and lung tissues than those in the liver and heart. We hypothesize that this may be due to differences in the post-translational processing of the protein in these tissues. Mouse TGH mRNA and protein were expressed predominantly in the liver, with lower levels in adipose, kidney and heart (Fig. 2.5). TGH transcripts and protein were not detected in brain, spleen or skeletal muscle. These results agree with previous studies that have detected the highest level of rat carboxylesterase transcripts and protein in the liver (27, 28). In addition, lower levels of rat carboxylesterase transcripts and protein have been detected in heart (28), kidney (29), and adipose tissues (30).

Considerable carboxylesterase activity exists in the rat small intestine (28). The TGH protein was previously detected in the small intestine of the rat (9). We investigated how the expression of

TGH is regionally distributed within the murine small intestine. TGH was most abundantly expressed in duodenal/jejunal sections of the small intestine and was undetectable in the more distal sections of the small intestine (Fig. 2.6).

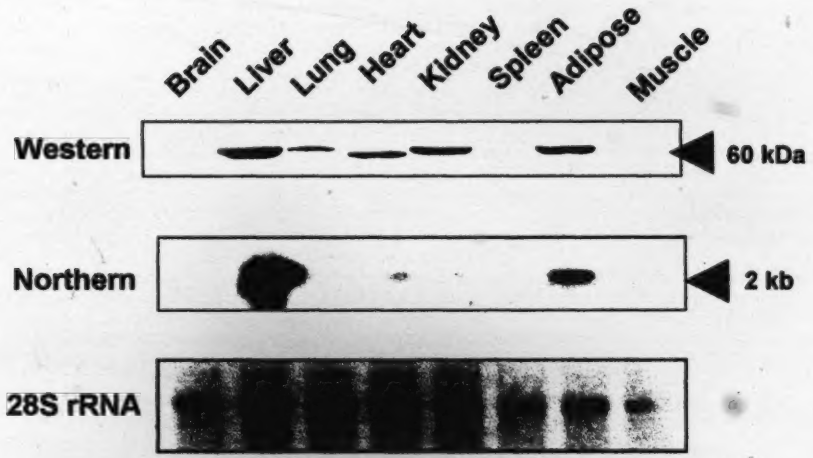


Figure 2.5: Tissue expression profile of the murine TGH message and protein.
For Northern analysis, 20μg total RNA was analyzed, as described in Sec. 2.2.5. For Western analysis, 25μg of microsomal protein was analyzed as described in Sec. 2.2.8.

Figure 2.6: Intestinal expression profile of the mouse TGH mRNA and protein. The small intestine from a mouse was sectioned into eleven ~3 cm sections. For Northern analysis, 20μg from each section was analyzed as described in Sec. 2.2.5. For Western blot analysis, 25μg small intestinal section homogenate protein was analyzed as described in Sec. 2.2.8. In addition, liver RNA and protein was also analyzed as a positive control.

2.4. Discussion

The mammalian carboxylesterases are a large multi-gene family proposed to play an important role in drug metabolism and detoxification by their ability to hydrolyze ester, thioester, or amide bonds (15, 31). Some carboxylesterases function to bind proteins and retain them within a specific subcellular compartment (30, 32, 33). Recently, some carboxylesterases were found which hydrolyzed lipid substrates. For example, the rat carboxylesterase 4 hydrolyzes acyl-CoA thioesters (34). Becker *et al.*, (35) claimed that a human carboxylesterase had acyl-CoA-cholesterol acyltransferase (ACAT) activity. However, subsequent papers have demonstrated that other proteins are responsible for catalyzing ACAT activity (36, 37). Some rat carboxylesterases have been implicated in the hydrolysis of retinyl esters (38). A rat carboxylesterase, identical to rat TGH, exhibited a 5-fold increase in cytosolic cholesterol ester hydrolysis activity when its cDNA was expressed in COS-7 cells (39). However, rat TGH protein should be targeted to the ER and combined with our inability to demonstrate CE hydrolysis by TGH, we are unable to confirm these results (40). Furthermore, the murine TGH is expressed in adipocytes and isolated adipocytes from HSL KO mice are deficient in CE activity (41), suggesting that if murine TGH had significant CE activity it would sufficiently compensate for the targeted deletion of HSL. More recently, a rat carboxylesterase cDNA that encodes rat TGH was expressed in McArdle RH7777 cells. The intracellular TG stores were depleted and the secretion of TG mass into the medium was increased by 25%, compared to empty vector transfected cells (11). Moreover, inhibition of hepatic lipolysis dramatically reduced the secretion of

TG and apoB by primary rat hepatocytes (12).

As shown in Fig. 2.2, the sequence of the rabbit carboxylesterase is 78% identical to the sequence of the murine TGH and secondary features of the TGH protein are conserved across species. Several molecular models have been constructed for lipases closely related to the ones with solved crystal structures. Therefore we constructed a model for the murine TGH protein (Fig. 2.3) based upon the crystal structure for the rabbit carboxylesterase (26). This model shows a similarity to the crystal structure of pancreatic lipase (42, 43). Each enzyme shares a similar three-dimensional fold called the α/β hydrolase fold (16, 44). In essence, it consists of a central β-sheet surrounded by a variable number of α-helices and accommodates a catalytic triad composed of serine, histidine and a carboxylic acid (16, 44). The modeled structure for TGH may be divided into three domains. The catalytic domain includes alpha helices 4, 5, 13 and 15 as well as beta-strands 7-9 and 12-13. These include the most highly conserved amino acid residues among orthologous carboxylesterases, suggesting that the catalytic function of these proteins is highly conserved. The regulatory domain is composed of alpha helices 10-12 and 16. A potential determinant of the affinity of TGH for lipids is a neutral lipid binding domain located on alpha helix 12. The FLXLXXXn motif is present in several proteins that bind lipids (17-21). The lipid binding domain shows less conservation among orthologous carboxylesterases and suggests that the variability of amino acids in this domain may be responsible for the differences in substrate preference and inhibition profiles of carboxylesterases from the

different species (Fig. 2.2). The alpha-beta domain is adjacent to the catalytic and regulatory domains and is composed of alpha helices 6-8, and beta strands 10-11 and 14-15.

Given their structural similarity, we can extrapolate site directed mutagenesis analysis of the human TGH to the murine TGH as well as the rabbit carboxylesterase (see Fig. 2.2 and 2.3). We have recently shown that mutation of any of Ser 221, Glu 354 and His 468 of the human TGH to Ala abolishes TGH esterase activity (45). Indeed, the role of these residues in catalysis is consistent with their orientation within the active site as suggested by the crystal structure of the rabbit carboxylesterase (26) and the model for the murine TGH structure (Fig. 2.3). This permits us to present a model for the catalytic activity of the murine TGH enzyme (Fig. 2.7). Briefly, Glu353 forms a low energy hydrogen bond with His 466 that facilitates proton transfer from His466 to Ser222 and nucleophilic attack by the Ser222 residue through the stabilization of the tetrahedral intermediate. In addition weak hydrogen bonds between peptide N-H bonds of Gly141 and Gly142 act to stabilize the tetrahedral intermediate. In the second stage, a proton is removed from His466, thereby forming an acyl-enzyme complex at Ser222. Once the alcohol product is released and substituted by a water molecule within the active site, a hydrolytic step occurs to release the fatty acid.

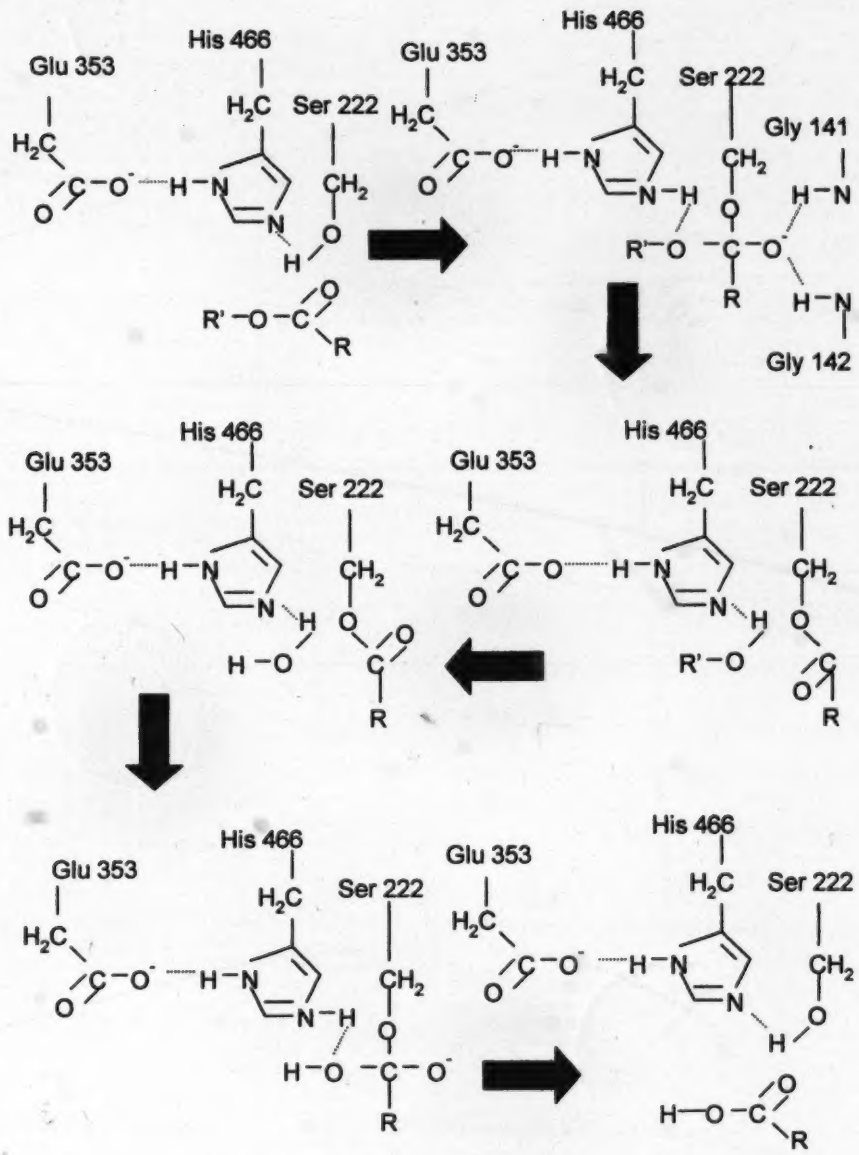


Figure 2.7- Proposed catalytic mechanism of murine TGH.

Several additional features were identified within the TGH amino acid sequence. Four cysteine residues were identified. The crystal structure indicates that Cys87 and Cys116 form one disulfide bridge and Cys273 and Cys284 form another disulfide bridge that stabilize the protein structure. Two consensus sites for N-linked glycosylation have been identified at Asn 79 and 489. The X-ray crystal structure of the orthologous rabbit carboxylesterase and DIG glycan detection assay of the human TGH indicate that the mature TGH protein was glycosylated (26, 45). However, mutation of Asn 79 to Ala reduced human TGH activity only 20%, indicating that glycosylation was not necessary for esterase activity (45). The C-terminal HXEL motif present at the C-terminus of several esterases has been proposed to have a function in the localization to the ER (24). Previously, it was demonstrated that the deletion of the similar C-terminal HIEL sequence from the human TGH protein did not significantly reduce localization of human TGH to the microsomal fraction of Sf9 insect cells (45). This result suggested that additional unidentified sequences have the ability to retain TGH in the microsomal fraction of Sf9 cells. However, more recent data suggests that deletion of the C-terminal HIEL sequence from human TGH causes the expressed protein to be secreted into the media of McArdle RH7777 cells (46). Therefore the HXEL sequence is a functional ER-retrieval sequence in mammalian cells.

If TGH activity is a component of the pathway for the assembly and secretion of apoB-containing lipoprotein particles, then it would be anticipated that TGH would be expressed in tissues that have the ability to synthesize and secrete apoB containing lipoproteins. The high level of TGH expression within the liver is

consistent with our hypothesis that TGH is involved in the mobilization of intracellular TG stores for VLDL secretion (11). Given the high capacity of the liver to secrete VLDL-TG, a high level of TGH expression within the liver would be required. The kidney (47, 48) and heart (49, 50) have been shown to express MTP and possess the ability to secrete apoB-containing lipoproteins. However, a role for TGH in the secretion of TG-rich lipoproteins from these tissues remains to be demonstrated. Adipose tissue forms large intracellular TG droplets. Hormone sensitive lipase is involved in the mobilization of fatty acids from adipocyte TG stores. However, targeted deletion of the hormone sensitive lipase gene only eliminated a portion of the basal TG lipase activity of the adipocyte (41). The expression of TGH in adipose tissue makes the potential involvement of TGH in the mobilization of fatty acids from adipocyte TG stores an intriguing possibility (see Chapter 5). In addition, several cDNAs and ESTs encoding the murine TGH have been cloned from different tissues. In addition to the tissues where we detected TGH transcripts by Northern analysis, there is evidence for TGH expression in the mammary gland, skin, olfactory epithelium, salivary gland, eyeball, testis, ovary and uterus. A role for carboxylesterases in the fertility of a diverse group of organisms has been suggested (51, 52) though a potential function for TGH in the other tissues is more obscure.

Carboxylesterase activities have been previously demonstrated in the brain (53), spleen (53) and lung (54). We did not detect TGH expression in brain, spleen or skeletal muscle and suggest that the carboxylesterase activities in these tissues were likely due to the expression of other carboxylesterase isoforms. In

the lung, we did not detect the murine TGH mRNA, but we did detect a signal by Western blot. The murine TGH mRNA level in the lung may be below our ability to detect it by Northern blot. Alternatively, a serine esterase with a high degree of identity to the mouse TGH cDNA was cloned from human alveolar macrophages and the undetectable levels of the transcript and low levels of the protein in murine lung may be due to the presence of alveolar macrophages that would be harvested together with the lung tissues (55). The finding of high levels of TGH expression within duodenal/jejunal sections of the small intestine corresponds to a previous report that microsomal triglyceride transfer protein mRNA and protein expression was greatest in the duodenum and jejunum of the hamster small intestine (56). We hypothesize that the expression pattern of TGH in the murine small intestine suggests a role for TGH in intestinal TG-rich, apoB containing lipoprotein secretion. It has previously been shown that TG stored within the enterocyte undergoes a lipolysis re-esterification cycle prior to TG secretion as an apoB particle (57) much like has been observed in the liver (6-8).

Given the structural similarity of TGH to that of known lipases (16), as well as the established function of TGH in the mobilization of stored TG (11) and its inhibition by E600 (12) and tetrahydrolipstatin (9) we have ample evidence to classify TGH as a lipase. Furthermore, the expression of TGH in a variety of tissues that store and secrete TG and have a high lipolytic capacity fits our hypothesis that TGH could be involved in the mobilization of intracellular stored TG.

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CHAPTER 3

THE STRUCTURE AND TARGETED DELETION OF THE MURINE TGH GENE

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and
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3.1. Introduction

The results from Chapter 2 indicate that the murine TGH is most closely related to other carboxylesterase enzymes and its structure and activity demonstrate that it may also be classified as a lipase (1-3). The mammalian carboxylesterases (E.C. 3.1.1.1) are serine esterases that comprise a multigene family of enzymes that are implicated in the hydrolysis of molecules containing ester bonds. Multiple forms of carboxylesterases have been identified in several mammalian tissues and some have been shown to have distinct biochemical, immunological, and genetic properties [for review see (1) and references therein]. Currently, information regarding the functions and regulation of individual carboxylesterases is very limited. Recent developments in molecular biology have identified several unique carboxylesterases. This information will facilitate detailed characterization of the regulation of individual carboxylesterase genes and the biological activities of the encoded enzymes. In order to further characterize a novel murine carboxylesterase, TGH, the murine TGH gene was isolated and its exon-intron organization determined. A promoter sequence was identified and analyzed in order to characterize the regulation of the TGH gene. A targeting vector was constructed in order to examine the effect of TGH deletion *in vivo*. Additionally, the characterization of the TGH gene and prospects for the phenotype of its targeted deletion are discussed in the context of the recent completion of the mouse genome sequencing project.

3.2 Materials and Methods

3.2.1 *Reagents*

All reagents and chemicals used are listed in Chapter 2 (see 2.2.1).

3.2.2 *DNA isolation and Southern analysis*

BAC DNA was isolated using a Qiagen midi-prep kit (Tip 100), plasmid DNA was isolated with a Wizard miniprep kit (Promega), and genomic DNA was extracted by proteinase K digestion of 129J mouse livers, followed by precipitation of the genomic DNA. To perform Southern blot analysis, DNA was separated by agarose gel electrophoresis and transferred to a Hybond-N+ nylon membrane (Amersham) using a vacuum blotter (Biorad) according to the manufacturer's instructions. Pre-hybridization and hybridization steps were carried out in 50% formamide, 5X SSC, 5X Denhardt's Solution, 0.5% SDS, 100 $\mu g\ ml^{-1}$ sheared salmon testes DNA (Sigma) and $1.5x10^6$ cpm ml^{-1} of probe at 42°C. Membranes were washed in conditions of low (1 x SSC, 0.1% SDS at room temp.) and high (0.1 X SSC, 0.1% SDS at 59°C) stringency. Blots were exposed to a phosphorimager screen and scanned using a Storm 540 phosphorimager (Molecular Dynamics).

3.2.3 *TGH-Specific PCR Primer Sequences*

All primers were synthesized at the DNA core facility, University of Alberta using a 394 DNA/RNA synthesizer (Applied Biosystems). MOUSE TGH: ATGCGCCTCTACCCTCTGATATG; MOUSE TGH': CCTCCTGATCAGAGCTCAACATG; Ex2F: CTGGGGAAGTATGTC AATTTGG; Ex2R: CAGGACTTTGCCTTTAACAGTGTTC; Ex3F:

CAGAGCTCTTCACCAACAGG; Ex3R: AGAGCACCTGCCCTCCAA C; Ex4F: AATGTGGTGGTGACCATTCAG; Ex5F: ACTGGGGATGA ACACAGTCG; Ex5R: AAGTGACCCCAGTTTCCCCG; Ex6F: CACTGCTGCTCTGATTACAACAG; Ex7F: GTGTAAAACTACCA CATCAGCTGTT; Ex7R: CCCAGAAAGAGTAGCAACCAG; Ex8F: AAACTGGACTTACTTGGAAATCC; Ex8R: TTGGATTTCCAAGTAA GTCCAG; Ex9F: CTCCCTACTGTGATTGATGGAGTAG; Ex9R: CCATCAATCACAGTAGGGAGG; Ex10F: ATTCTCTCTTGTGGA AGTCCTACC; Ex10R: GCCTTCAGCGAGTGGATAGC; Ex11F: GACCTGTTCCAGGACTTGATGG; Ex11R: GCGACCACTGGAATC ATATTC; Ex12F: TATCGGCCATGAGACCCAAGG; Ex12R: ACTGCCTTGGGTCTCATGGC; Ex13F: GACCACCTCAGCAAGAT GG; Ex13R: CACCATCTTGCTGAGGTTGGTC; Ex14R: TCTGGTCATATTCTGGCCAGTG; TGP-2': TAGTCCACCTCCATG GATCC; TGP-3': AGTGAGGGCCACACCACTTT;

3.2.4 *Cloning of the murine TGH gene*

Using the murine TGH cDNA sequence, 129 J murine genomic DNA packaged into a BAC library (Research Genetics) was screened using PCR. To confirm the presence of TGH sequence in isolated clones, BAC DNA was digested using *Bam*HI or *Eco*RI and subjected to Southern blot analysis with a ^{32}P-murine TGH cDNA probe.

3.2.5 *Gene structure*

BAC DNA was digested with *Bam*HI, *Eco*RI, and *Hind*III restriction enzymes and fragments that contained exonic sequences were identified by Southern blot using a radiolabeled murine TGH cDNA

or exon-specific oligonucleotide probes. Fragments that hybridized were subcloned and sequenced. Using primers based on the murine TGH cDNA sequence and designed with regard to the structure of the human carboxylesterase gene (4,5), exon-intron boundary sequences were determined. PCR amplification of murine TGH gene introns were carried out as follows: Intron 1 (primers MouseTGH-Ex2R) 95°C 1 min., 60°C 1 min., 72°C 3 min. for 40 cycles. Intron 2 (primers Ex2F-Ex3R) 95°C 1 min., 57°C 1 min., 72°C 3 min. for 40 cycles. Intron 3 (primers Ex3F-TGP-2') 95°C 1 min., 58°C 1 min., 72°C 3 min. for 40 cycles. Intron 4 (primers Ex4F-Ex5R) 95°C 1 min., 65°C 1 min., 72°C 3 min. for 40 cycles. Intron 5 (primers Ex5F-TGP-3') 95°C 1 min., 55°C 1 min., 72°C 3 min. for 40 cycles. Intron 6 (primers Ex6F-Ex7R) 95°C 1 min., 57°C 1 min., 72°C 3 min. for 40 cycles. Intron 7 (primers Ex7F-Ex8R) 95°C 1 min., 57°C 1 min., 72°C 3 min. for 40 cycles. Intron 8 (primers Ex8F-Ex9R) 95°C 1 min., 52°C 1 min., 72°C 3 min. for 35 cycles. Intron 9 (primers Ex9F-Ex10R) 95°C 1 min., 59°C 1 min., 72°C 3 min. for 40 cycles. Intron 10 (primers Ex10F-Ex11R) 95°C 1 min., 57°C 1 min., 72°C 3 min. for 35 cycles. Intron 11 (primers Ex11F-Ex12R) 95°C 1 min., 60°C 1 min., 72°C 3 min. for 35 cycles. Intron 12 (primers Ex12F-Ex13R) 95°C 1 min., 64°C 1 min., 72°C 3 min. for 35 cycles. Intron 13 (primers Ex13F-Ex14R) 95°C 1 min., 60°C 1 min., 72°C 3 min. for 35 cycles. PCR products were cloned into pCR2.1-Topo (Invitrogen), following the manufacturer's protocol. Cloned plasmid DNA was sequenced as described previously and intron/exon boundaries identified by comparison to the murine TGH cDNA.

3.2.6 *Cloning of the murine TGH promoter*

Genomic sequences containing the putative proximal mouse TGH promoter were identified by southern blot analysis of BAC clone 313P24 that contains the complete murine TGH gene. Briefly, BAC DNA was digested with *Eco*RI and fragments that contained exon 1 were identified by Southern blot using an exon 1 specific probe. A single 6 kb DNA fragment hybridized. This fragment was subcloned and sequenced by gene walking using overlapping primers to determine sequences upstream of the transcriptional start site that was determined previously (see 2.3.1). The sequence of the murine TGH promoter was confirmed by sequencing of both strands of the DNA. A genomic fragment encoding the murine TGH promoter region spanning -547 and +113 was cloned into the T/A cloning site of pCR2.1 TOPO (Invitrogen) according to the manufacturer's instructions to create pCR (-547/+113). The promoter region was excised from pCR 2.1 TOPO by restriction digestion with *Kpn*I and *Xba*I and directionally inserted into the luciferase reporter vector, pGL_3Basic (Promega) to generate -542Luc.

3.2.7 *Cloning of the murine TGH targeting vector*

Starting from BAC clone 313P24 derived from the 129J mouse ES cell genomic DNA library (Research Genetics) and containing the entire murine TGH gene, a 5.7 kb *Bam*HI fragment and a 3 kb *Eco*RI-*Kpn*I fragment were separately cloned into pBluescript SK-. A targeting vector was constructed by inserting a thymidine kinase cassette (TK) at an *Rsr*II site and a neomycin resistance gene (neo) at an *Asc*I site (kind gifts of Dr. L. Agellon, University of Alberta).

The short arm of the targeting construct was generated by directionally inserting the 3kb *Eco*RI-*Kpn*I fragment at *Eco*RI and *Kpn*I sites between the TK and neo cassettes in the vector. The long arm of the targeting construct was generated by inserting the 5.7 kb *Bam*HI fragment at a *Bam*HI site immediately following the neo cassette in the vector. The orientation of the various fragments in the targeting vector was confirmed by restriction mapping and sequencing (not shown).

3.2.8 *Database searches*

BLAST was used to search the Genbank and EMBL databases. PSI-Pred and Predict Protein servers were used to analyze murine carboxylesterases for secondary structure features. Potential binding sites for transcription factors in the TGH promoter were identified by searching the TRANSFAC database with promoter sequences using the Matinspector program (6).

3.3 Results

3.3.1 *Cloning of the murine triacylglycerol hydrolase gene and identification of intron/exon boundaries*

The structure of the murine TGH gene was determined from 129 J mouse genomic DNA packaged into a BAC clone. Previously, the presence of the TGH sequence in the BAC clone was confirmed by restriction digestion of the BAC clone DNA using *Bam*HI or *Eco*RI and subjected to Southern blot analysis using the ^{32}P-murine TGH cDNA probe. Structure of the clone was confirmed by comparison with a 129 J mouse genomic DNA restriction pattern using the same ^{32}P-murine TGH cDNA probe. Using the structure of the human carboxylesterase gene as a guide for the location of intron-exon boundaries (4, 5), PCR primers were designed based on the murine TGH cDNA sequence and exon-intron boundaries were determined by PCR of the BAC clone. Furthermore restriction fragments spanning the entire murine TGH gene were identified within the BAC clone, subcloned and sequenced. The sequences of the exons match those of the murine TGH cDNA (see 2.3.1). A restriction map of the major sites used for cloning of the murine TGH gene is presented (Fig. 3.1). The murine TGH gene spans approximately 35 kb and contains 14 small exons (Table 3.1). Each intron sequence begins with the dinucleotide GT and ends with the dinucleotide AG, in agreement with the consensus sequence for splice junctions of eukaryotic genes (7). Recently the sequencing of the mouse genome was completed (8). The sequences of the individual exons and size of the introns in the murine TGH presented herein and by the mouse genome database are

consistent (Table 3.2). In addition, the sequences of introns at the splice junctions and the restriction sites within the murine TGH gene are identical to those for the murine TGH gene sequenced by the mouse genome project (not shown). Therefore we conclude that the structure of the murine TGH gene and the corresponding restriction map for the murine TGH gene is accurate. Furthermore, the location of intron-exon boundaries, size of the murine TGH gene and its individual exons, are similar to the previously published human TGH gene (Table 3.3) (4, 5). Therefore the organization of the TGH gene is evolutionarily conserved in mice and humans respectively.

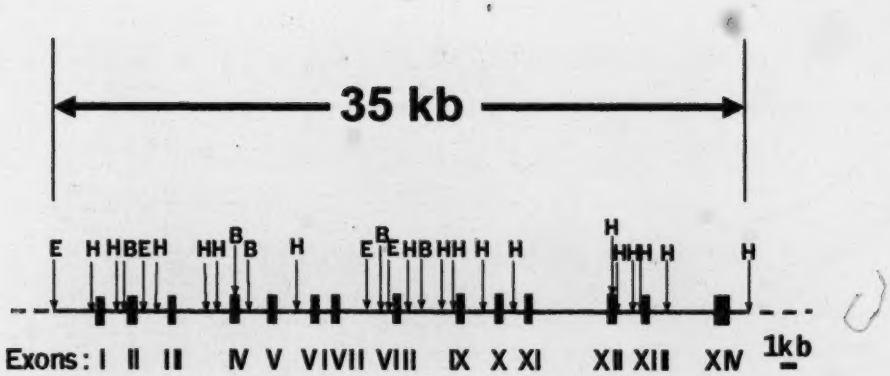

Figure 3.1- The structure and restriction map of the murine triacylglycerol hydrolase gene.
Numbered blue boxes and long red lines indicate exons and introns respectively. The action sites of restriction enzymes used for sub-cloning are indicated. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII.

Table 3.1 Exon-intron junctions of the murine triacylglycerol hydrolase gene. Exon sequences are indicated in upper case and intron sequences are in lower case.

No.	Exon (bp)	Intron (kb)	5' splice donor	3' splice acceptor
1	112	2.2	CAGCTTGGGgtaagt	caacagGGTACCCAT
2	205	1.8	CCCGCCTATgttaag	ttccagGTGCTCTCA
3	145	2.9	AGACTACCAgtacgt	gtccagGTGATGGTG
4	134	1.6	ATTCTTCAGgtaaga	ttgcagCACTGGGGA
5	154	1.7	TCTGTTCTTgtaagt	tgccagGTCTTGTCT
6	105	1.0	ATTGCTGGTgtaagt	tggcagCTGGTTGCT
7	105	3.2	ACTAAAATTGgtaagt	tcgtagAATCTTTTT
8	39	3.5	CCAAAAGAGgtaagg	ctctagAGCTATCCC
9	141	2.0	ATTCCAACGgtgaga	taacagCTTATGGGC
10	81	1.0	CCAACACTTgtaaga	tttcagAAAATCTCT
11	148	4.0	GTCACAGAGgtgagt	ccgcagATGCTGGAG
12	132	1.6	TTTTAAAAGgtaatg	atacagATGGTGCCT
13	73	3.5	TCGGAATGGgtgatc	tcacagGAACCCCAA
14	388			

Table 3.2- Comparison of murine triacylglycerol hydrolase gene (16) with the murine triacylglycerol hydrolase gene from the map of the mouse genome (8)

No.	Published Exon(bp)	Database Exon(bp)	Published Intron(bp)	Database Intron(bp)
1	112	112	~2200	2586
2	205	205	~1800	1971
3	145	145	~2900	3061
4	134	134	~1600	1565
5	154	154	~1700	1624
6	105	105	~1000	947
7	105	105	~3200	3397
8	39	39	~3500	3353
9	141	141	~2000	1783
10	81	81	~1000	987
11	148	148	~4000	3879
12	132	132	~1600	1226
13	73	73	~3500	3282
14	368	368		

Table 3.3- Comparison of murine triacylglycerol hydrolase gene (16) with the human triacylglycerol hydrolase gene (5)

No.	Murine Exon(bp)	Human Exon(bp)	Murine Intron(kb)	Human Intron(kb)
1	112	119	~2.2	~4.0
2	205	205	~1.8	~2.6
3	145	145	~2.9	~2.4
4	134	134	~1.6	~1.9
5	154	154	~1.7	~0.8
6	105	108	~1.0	~0.7
7	105	105	~3.2	~2.6
8	39	39	~3.5	~4.4
9	141	141	~2.0	~2.2
10	81	84	~1.0	~0.2
11	148	148	~4.0	~4.2
12	132	132	~1.6	~3.2
13	73	73	~3.5	~0.5
14	368	379		

3.3.2 *The murine TGH gene is located on chromosome 8 in the carboxylesterase gene cluster.*

Previous studies have mapped the human carboxylesterase gene to chromosome 16 at 16q13-q22.1 (9, 10). This region is syntenic to a region of mouse chromosome 8 at 8C5 (9). Previously we found that BAC clone 313P24 containing the entire murine TGH gene also contained portions of the Es22 and Es1 carboxylesterase genes, suggesting the close linkage of these genes. Es22 (11, 12) and Es1 (13, 14) have been previously mapped to chromosome 8. The completion of the mouse genome sequencing project unambiguously demonstrated that the murine TGH gene was located on the minus strand of chromosome 8 at 8C5 in a cluster of carboxylesterase genes (8). The entire cluster spans 260.6 kb and is composed of six genes (Fig. 3.2). These genes encode proteins with significant identity to the other carboxylesterases, four of which have been previously studied (12, 14-16). The proteins encoded by Es 22 and CES1 are most closely related to TGH having 76% identity to TGH, while Es1 is the least closely related having only 69% identity to TGH. Two novel genes were identified. LOC234564 has 70% identity to TGH and the 2310039D24 Rik gene does not code for a complete carboxylesterase protein. 2310039D24 Rik has identity to the 305 C-terminal residues of TGH, but does not contain the GXSXG catalytic serine motif. If this protein is expressed in a cell, it would not be expected to have esterase activity and its putative function is obscure. The remaining five genes in the cluster have significant homology to TGH (Fig. 3.3). The Ser222, Glu 353 and His466 catalytic residues were conserved in all of these carboxylesterases, suggesting that they

have esterase activity (1). Furthermore, it appeared that most of the secondary structure features were also conserved (Fig. 3.3) suggesting that the tertiary structure of the proteins encoded by genes within the carboxylesterase cluster would be expected to be very similar (2, 17). The size and location of exon-intron boundaries of the clustered carboxylesterase genes is similar to TGH (Table 3.4 and 3.5). Each gene spans approximately 20-35 kb and is composed of 13 or 14 small exons.

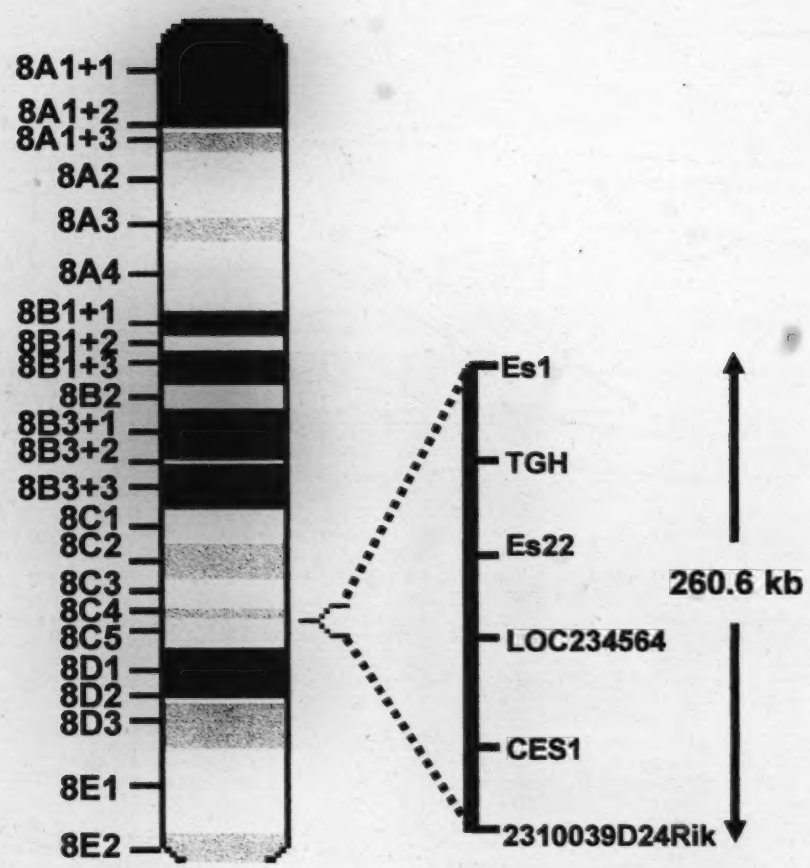

Figure 3.2- Chromosomal localization of the triacylglycerol hydrolase gene and the murine carboxylesterase gene cluster to chromosome 8C5.

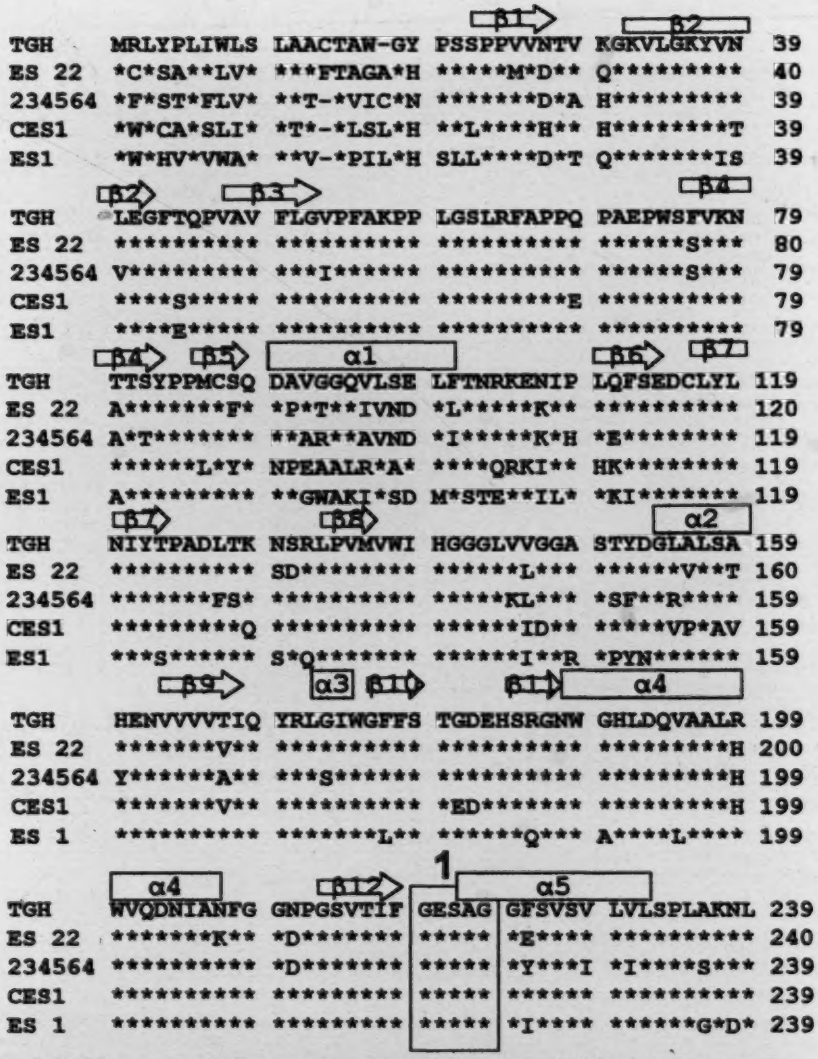
β1
β2
TGH MRLYPLIWLS LAACTAW-GY PSSPPVVNTV KGKVLGKYVN 39
ES 22 *C*SA**LV* ***FTAGA*H *****M*D** Q********* 40
234564 *F*ST*FLV* **T-*VIC*N *******D*A H********* 39
CES1 *W*CA*SLI* *T*-*LSL*H **L****H** H*********T 39
ES1 *W*HV*VWA* **V-*PIL*H SLL****D*T Q*******IS 39
β2
β3
β4
TGH LEGFTQPVAV FLGVPFAKPP LGSLRFAPPQ PAEPWSFVKN 79
ES 22 ********** ********** ********** ******S*** 80
234564 V********* ***I****** ********** ******S*** 79
CES1 ****S***** ********** *********E ********** 79
ES1 ****E***** ********** ********** ********** 79
β4
β5
α1
β6
β7
TGH TTSYPPMCSQ DAVGGQVLSE LFTNRKENIP LQFSEDCLYL 119
ES 22 A*******F* *P*T**IVND *L*****K** ********** 120
234564 A*T******* **AR**AVND *I*****K*H *E********* 119
CES1 ******L*Y* NPEAALR*A* ****QRKI** HK********* 119
ES1 A********** **GWAKI*SD M*STE**IL* *KI******* 119
β7
β8
α2
TGH NIYTPADLTK NSRLPVMVWI HGGGLVVGGA STYDGLALSA 159
ES 22 ********** SD********* ******L*** ******V**T 160
234564 ********FS* ********** *****KL*** *SF**R**** 159
CES1 *********Q ********** ******ID** *****VP*AV 159
ES1 ***S****** S*Q******* ******I**R *PYN****** 159
β9
α3
β10
β11
α4
TGH HENVVVVTIQ YRLGIWGFFS TGDEHSRGNW GHLDQVAALR 199
ES 22 *******V** ********** ********** *********H 200
234564 Y******A** ***S****** ********** *********H 199
CES1 *******V** ********** *ED******* *********H 199
ES 1 ********** *******L** ******Q*** A****L**** 199
1
α4
β12
α5
TGH WVQDNIANFG GNPGSVTIF GESAG GFSVSV LVLSPLAKNL 239
ES 22 *******K** *D******* ***** *E**** ********** 240
234564 ********** *D******* ***** *Y***I *I****S*** 239
CES1 ********** ********* ***** ****** ********** 239
ES 1 ********** ********* ***** *I***** ******G*D* 239

Fig. 3.3- Alignment of the murine carboxylesterases in the chromosome 8 gene cluster. TGH single letter coding sequence is shown at the top. Sequences for Es 22, a carboxylesterase gene LOC234564, CES1 and Es 1 are aligned below. Amino acid residues identical to TGH are represented by an asterix. Non-aligned residues are indicated by the single letter amino acid sequence. Thick yellow arrows above the sequence are regions forming beta sheets in the TGH protein. Blue rectangles indicate regions forming alpha helices in the TGH protein. **1**, GXSXG catalytic serine motif **2**, catalytic glutamic acid **3**, catalytic histidine **4**, putative neutral lipid binding sequence.

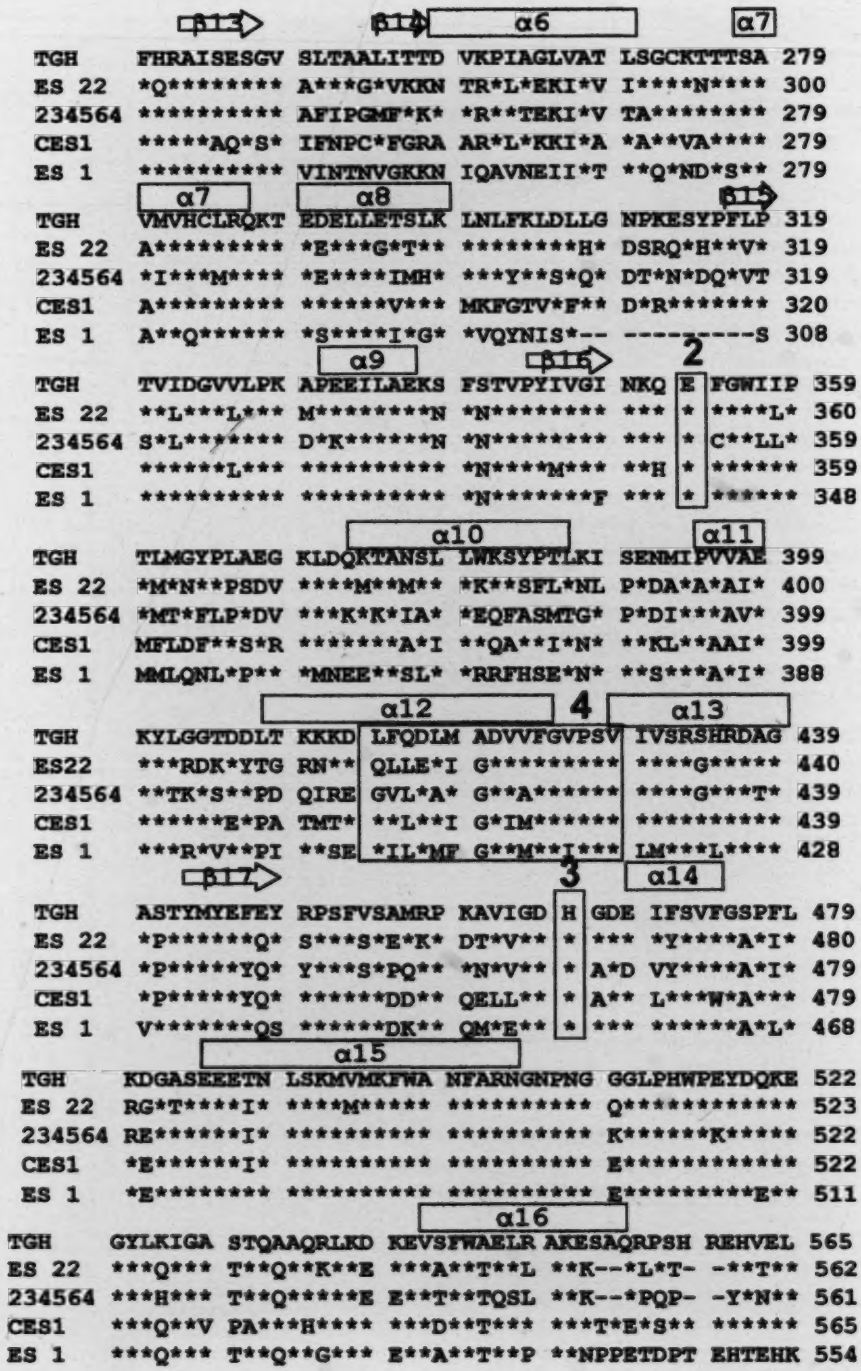
β13
β14
α6
α7
TGH FHRAISESGV SLTAALITTD VKPIAGLVAT LSGCKTTTSA 279
ES 22 *Q********* A***G*VKKN TR*L*EKI*V I****N**** 300
234564 ********** AFIPGMF*K* *R**TEKI*V TA******** 279
CES1 *****AQ*S* IFNPC*FGRA AR*L*KKI*A *A**VA**** 279
ES 1 ********** VINTNVGKKN IQAVNEII*T **Q*ND*S** 279
α7
α8
β15
TGH VMVHCLRQKT EDELLETSLK LNLFKLDLLG NPKESYPFLP 319
ES 22 A********* *E***G*T** ********H* DSRQ*H**V* 319
234564 *I***M**** *E****IMH* ***Y**S*Q* DT*N*DQ*VT 319
CES1 A********* ******V*** MKFGTV*F** D*R******* 320
ES 1 A**Q****** *S****I*G* *VQYNIS*-- ----------S 308
α9
β16
2
TGH TVIDGVVLPK APEEILAEKS FSTVPYIVGI NKQ E FGWIIP 359
ES 22 **L***L*** M********N *N********* *** * ****L* 360
234564 S*L******* D*K*******N *N********* *** * C**LL* 359
CES1 ******L*** ********** *N****M*** **H * ****** 359
ES 1 ********** ********** *N********F *** * ****** 348
α10
α11
TGH TLMGYPLAEG KLDQKTANSL LWKSYPTLKI SENMIPVVAE 399
ES 22 *M*N**PSDV ****M**M** *K**SFL*NL P*DA*A*AI* 400
234564 *MT*FLP*DV ***K*K*IA* *EQFASMTG* P*DI***AV* 399
CES1 MFLDF**S*R *******A*I **QA**I*N* **KL**AAI* 399
ES 1 MMLQNL*P** *MNEE**SL* *RRFHSE*N* **S***A*I* 388
α12
4
α13
TGH KYLGGTDDLT KKKD LFQDLM ADVVFGVPSV IVSRSHRDAG 439
ES22 ***RDK*YTG RN** QLLE*I G********* ****G***** 440
234564 **TK*S**PD QIRE GVL*A* G**A****** ****G***T* 439
CES1 ******E*PA TMT* **L**I G*IM****** ********** 439
ES 1 ***R*V**PI **SE *IL*MF G**M**I*** LM***L**** 428
β17
3
α14
TGH ASTYMYEFEY RPSFVSAMRP KAVIGD H GDE IFSVFGSPFL 479
ES 22 *P******Q* S***S*E*K* DT*V** * *** *Y****A*I* 480
234564 *P*****YQ* Y***S*PQ** *N*V** * A*D VY****A*I* 479
CES1 *P*****YQ* ******DD** QELL** * A** L***W*A*** 479
ES 1 V*******QS ******DK** QM*E** * *** ******A*L* 468
α15
TGH KDGASEEETN LSKMVMKFWA NFARNGNPNG GGLPHWPEYDQKE 522
ES 22 RG*T****I* ****M***** ********** Q********** 523
234564 RE******I* ********** ********** K******K***** 522
CES1 *E******I* ********** ********** E********** 522
ES 1 *E******** ********** ********** E**********E** 511
α16
TGH GYLKIGA STQAAQRLKD KEVSFWAELR AKESAQRPSH REHVEL 565
ES 22 ***Q*** T**Q**K**E ***A**T**L **K--*L*T- -**T** 562
234564 ***H*** T**Q*****E E**T**TQSL **K--*PQP- -Y*N** 561
CES1 ***Q**V PA***H**** ***D**T*** ***T*E*S** ****** 565
ES 1 ***Q*** T**Q**G*** E**A**T**P **NPPETDPT EHTEHK 554

Table 3.4- Organization of aligned exons of genes within the murine carboxylesterase gene cluster on chromosome 8

No.	TGH (bp)	2310039 D24Rik	CES1 (bp)	LOC 234564	Es22 (bp)	Es1 (bp)
1	112		92	63	172	65
2	205		205	205	208	205
3	145		145	145	145	145
4	134		134	134	134	134
5	154		154	154	154	154
6	105	52	105	105	105	105
7	105	105	105	105	105	111
8	39	39	39	39	39	-
9	141	141	141	141	141	141
10	81	81	81	81	81	81
11	148	148	148	148	148	148
12	132	132	132	132	132	132
13	73	73	73	73	73	73
14	368	354	693	359	355	334

Table 3.5- Organization of introns of genes within the murine carboxylesterase gene cluster

No.	TGH (kb)	2310039 D24Rik	CES1 (kb)	LOC 234564	Es22 (kb)	Es1 (kb)
Total	31.6	11.4	34.3	23.1	27.9	30.9
1	2.5		1.9	4.2	5.3	0.3
2	2.0		1.3	1.0	2.3	3.0
3	3.1		2.5	2.1	2.1	2.9
4	1.6		2.5	1.7	1.7	1.2
5	1.6		1.5	1.9	2.4	2.7
6	1.0	0.7	0.9	0.6	0.8	1.8
7	3.4	1.2	2.8	0.9	1.8	8.9
8	3.4	2.7	2.9	0.4	1.8	0.8
9	1.8	1.3	1.7	2.3	1.4	0.8
10	1.0	0.3	0.9	0.2	0.2	2.4
11	3.9	3.4	10.2	4.6	5.2	3.4
12	1.3	0.9	1.0	1.3	1.3	1.3
13	3.3	0.2	2.8	0.3	0.3	-

3.3.3 *Cloning of the murine TGH promoter and analysis of putative transcription factor binding sites.*

To investigate the transcriptional regulation of the murine TGH gene, a 6 kb DNA fragment derived from a BAC clone, previously demonstrated to contain the entire murine TGH gene has been sequenced. This DNA fragment contains exon 1, most of intron 1 and extends 3 kb upstream of the transcription start site and presumably contains the promoter. Herein we present 542 bp of the 5' sequence flanking exon 1 (Fig. 3.4). This sequence is 59% identical to orthologous rat (18) and 46% identical to human (5) promoters (Fig. 3.5). No TATA box has been found to precede the transcription start site. Potential binding sites for transcription factors have been identified by searching the TRANSFAC database with the mouse sequence using the Matinspector program (6). These include three Sp1 binding sites, an NF-1 and three SRE-like sequences. Interestingly, several of these binding sites are also present in the human and rat TGH promoter sequences suggesting that we have cloned a functional promoter with evolutionarily conserved transcriptional regulatory patterns (Fig. 3.5).

```
                                                    NF-1
-542 ACCTAAGTAT GCACCGTGCC CAGCCAGGTC ACTTGGCAGG AATACTACAA

-492 ACTTAATTTA GGAATCCTCT AAAGGTTGGT AAGTGTGAGT TCCCTTCCTA

-442 ACTCGAGAGT ATGGATTCTA AGACATGCAG GTCCAGGAAG GCTGTGAAAT
                          SRE                   GRE
-392 GTGTCCGTCC TCACATCCCA GCTGAGCAGA GCTTTGTAGA AGGACCCCAG
                 SRE
-342 AACAACACTT CCCACTACCT GCAGGCACCT GGCTGCTGCT GTCTGCTCTT
           PPRE            NF-Y      GRE
-292 GAGAGGTCAG GGTGCACTGA ATTGAGGTGA GAGTGCTGCA GGGAGAGGTG
       C/EBP-β                          SRE
-242 CTTATGTAAG AAGCTGTTGG ATGGGTTTCT GGCCACTTGT AATCTGAGTA
       Sp1    Ap1                                   Sp1
-192 CTGGGCACTG ACTGTTCAGA TAAAAGTGGG TAAACTCCTA GTGGGCGTGG
                                                  site A
                          Ap1      Th1/E47
-142 CTTGGAGGGC CCAACTGACA CCCAGAGAGC TCTTTGGAAG GAAGAGTTTT
      site A                    site B

-92  TTTTTTTTTT TTTTTTTTTT TTTTTTTAAG CTTAGATTAC ATAACTGAGC

       C/EBP-β             Sp1                      +1 exon 1
-42 TGGTTGAGCA AGACTTAAGG AGGCGGGTCC CCTGGTCCAC AA cagaagca

+9 ttgctaaagc agcagatagc tcagagaccc acagagccct tgtccttccaca

+61 atg cgc ctc tac cct ctg ata tgg ctt tct ctt gct gcg tgc
     M   R   L   Y   P   L   I   W   L   S   L   A   A   C
+103 aca gct tgg g
      T   A   W
```

Figure 3.4- The 542-bp DNA sequence preceding the triacylglycerol hydrolase gene first exon (lower case) is shown. Putative binding sites for transcription factors are shown in bold. DNA sequences demonstrated to bind proteins(sites A and B) are underlined. The transcription start site is indicated by +1. The start codon is in bold with single letter amino acid coding for exon 1 shown underneath in capitals.

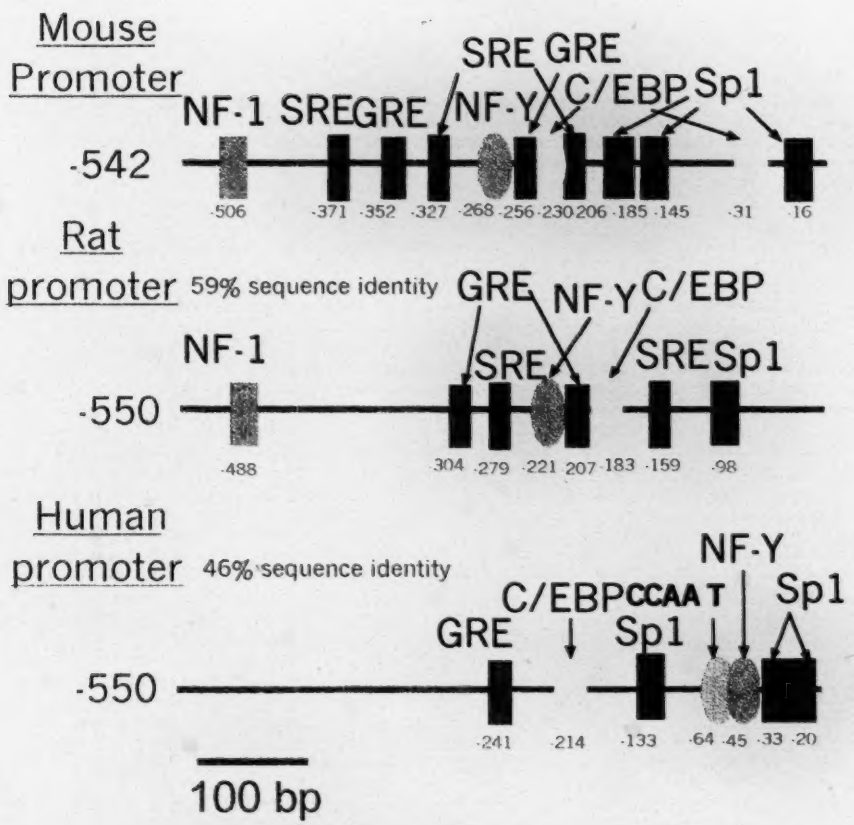

Figure 3.5- Putative Transcription Factor Binding Sites in the 5' flanking region of the mouse triacylglycerol hydrolase gene.
The promoter was analyzed using the TRANSFAC database. blue boxes represent sterol response elements, SRE; blue circles, CCAAT box; red boxes represent glucocorticoid response elements half-sites, GRE; Green boxes represent NF-1; green circles, NF-Y; Black boxes, Sp1; and Yellow circles represent CCAAT enhancer binding protein, C/EBP-β.

Site A Alignment:

		Sp1	
Mouse	-156	CCTAGT GGGCG TGGC TTGG	-138
Rat	-108	CTTGGT GGGCG TGGC TTGG	-89
Human	-140	GTGGGT GGGCG TGGC CTG-	-122

Site B Alignment:

		Th1/E47	
Mouse	-124	ACACCC AGAGAG CTCTTT	-107
Rat	-70	GCACCC AGAGAG CTCTTT	-60
Human	-109	AAGCCC AGGGAG ATCTGA	-92

Figure 3.6- Alignment of DN*ase* I protected sites A and B within the murine triacylglycerol hydrolase promoter with the corresponding sequences in the rat and human triacylglycerol hydrolase promoters. Previously, nuclear proteins footprinted a GC-rich region of the murine triacylglycerol hydrolase promoter: -156to -138 (site A) and -124 to -108 (site B).

3.3.4 *Construction of the murine TGH targeting vector*

The restriction map of the murine TGH gene (Fig. 3.1) permitted us to develop a strategy for the targeted deletion of the murine TGH gene. The vector replaced exon 1, which includes both the transcription and translation start sites, and the proximal promoter of the murine TGH gene with the neomycin resistance gene (Fig. 3.7). The vector was linearized with the *Not*I enzyme and electroporated into embryonic stem cells. Clones that survived antibiotic resistance screening were assayed for homologous recombination by Southern blot analysis of ES cell genomic DNA with either probe A and the *Xba*I enzyme or probe B and the *Pvu*II enzyme. Thus far, 640 clones have been screened without the identification of cells that underwent homologous recombination.

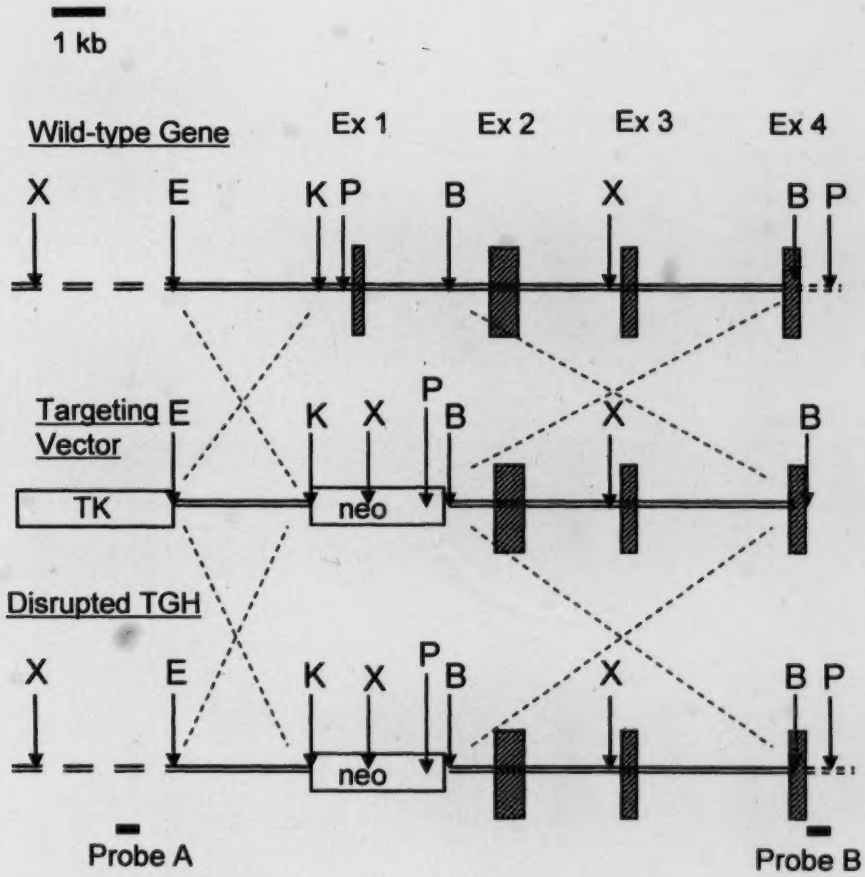

Fig. 3.7- Targeted Disruption of the murine triacylglycerol hydrolase gene.

Cross-hatched boxes and parallel lines indicate exons and introns, respectively. The action sites of major restriction enzymes are indicated by arrows. B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; P, *Pvu*II; X, *Xba*I.

3.4 Discussion

Previously, several carboxylesterase enzymes were identified and demonstrated to represent a multigene family, many of which have been genetically mapped to chromosome 8 (19). As a result of the mouse genome sequencing project it can now be unambiguously observed that TGH is a gene located on chromosome 8 at 8C5 in a cluster of six carboxylesterase genes. These six carboxylesterase genes are presumed to have originated from repeated gene duplications of a common ancestral gene that encoded a carboxylesterase (4, 19). Subsequently, evolutionary divergence of the structural genes into different groups occurred. Evidence for their common ancestral origin comes from the shared structure and size of these genes. Generally, these genes, including TGH, are composed of 14 exons and 13 introns, spanning 20-35kb (Table 3.3 and 3.4). Two notable exceptions to the rule are the genes at the 5' end and 3' end of this cluster. The 2310039 Rik gene is composed of only 9 exons and 8 introns. Though the protein encoded by these 9 exons is related to carboxylesterase it lacks domains of the carboxylesterase involved in catalysis, therefore if it is expressed it would not have esterase activity and its function would be obscure. The Es1 gene is composed of 13 exons and 12 introns. Interestingly the missing exon encodes a loop and beta sheet in the other carboxylesterase genes (Fig. 3.3) that forms the lower jaw of the active site cleft in the structure of the rabbit carboxylesterase (17). Therefore Es1 likely has a smaller active site cleft. In addition, the C-terminal ER-retrieval sequence is missing, causing the Es1 protein to be secreted, as has been demonstrated (20). These regions correspond to exons 2, 3, 4 and 5 of their

respective genes. Overall, it appears that the secondary structures and residues forming the catalytic domain of TGH are highly conserved among proteins encoded by the Es1, CES1, Es22 and LOC234564 genes (Fig. 3.3). The most diverged regions are located in the regulatory domain, especially in alpha helix 12 which has been proposed to function in binding neutral lipids in TGH (21). Therefore, the efficiency of the interfacial interaction between carboxylesterases and lipid substrates may be different. Most of the divergence in these genes appears to have occurred in the exons near the 3'-end of the gene.

Information on molecular mechanisms of transcriptional regulation of carboxylesterase genes is very limited. In order to study the transcriptional regulation of the TGH gene, we cloned the murine TGH promoter. We identified several consensus binding sites for transcription factors in the region immediately 5' to the transcriptional start sequence, but no TATA-box (Fig. 3.4). Interestingly, a unique 31bp poly-T repeat is located 65 bp upstream of the transcriptional start site. This poly-T repeat was not present in the human nor the rat TGH promoters (5, 18). Although unusual, this sequence did not impair promoter function, though this region of the promoter did not bind nuclear proteins (22). The putative promoter sequence was tested in order to determine whether the cloned sequence had promoter activity by designing fusion constructs of the 5'-flanking sequence linked to the luciferase gene. Indeed, the murine TGH promoter had significant promoter activity when expressed transiently in primary mouse hepatocytes (22).

The cloning of the murine TGH promoter was a major advance in our ability to study the regulation of the murine TGH

gene. In a previous study we observed increased expression of rat TGH mRNA and protein in liver at the time of weaning, coincident with enhanced ability to secrete VLDL (23). The murine TGH promoter was utilized to determine whether the increased expression of TGH seen at the time of weaning was linked to transcriptional regulation and to identify potential transcription factors and *cis*-acting DNA elements that might mediate the observed developmental expression of TGH in liver. Electrophoretic mobility shift assays demonstrated enhanced binding to the murine TGH promoter of hepatic nuclear proteins from 27-day old weaned mice compared to 7-day old suckling mice (22). DN*ase* I footprint analysis localized binding of nuclear proteins to two regions within the promoter: site A, which contains a Sp1 binding site and site B, which contains a degenerate E-box (Fig. 3.4). To elucidate the potential functional role of the footprinted regions and additional potential *cis* regulatory elements, subsequent nested deletions of the murine TGH promoter were constructed. Transient transfection into primary murine hepatocytes revealed a reproducible reduction of luciferase activity (~50%) upon deletion of the sequence spanning -154 to –117, suggesting that positive control elements reside within this segment (22). Competitive electromobility shift and supershift assays demonstrated that site A binds Sp1 and Sp3 transcription factors and transcriptional activation assays in Schneider SL-2 insect cells demonstrated that Sp1 was a potent activator of the TGH promoter (22). Sp1 is a ubiquitous nuclear protein that activates the transcription of a wide variety of genes (24). Since the TGH promoter lacks a TATA-box, TATA-less promoters have been shown to be particularly sensitive to regulation

by the Sp family of proteins (25-27). The ability of Sp factors to associate with individual components of the basal transcription machinery suggests that the Sp family of transcription factors regulate TATA-less promoters by bypassing selective steps in the assembly of the core transcriptional machinery. The ability of Sp transcription factors to regulate TATA-less promoters is also evident in the case of the TGH promoter.

Interestingly, sequence alignment of the 5' proximal promoters of the murine, rat and human TGH genes demonstrates that the regions that bind nuclear proteins corresponding to sites A and B sequences are evolutionarily conserved (Fig. 3.6). Indeed, others have observed that reporter constructs containing the conserved site A sequence activate transcription while their elimination reduces promoter activity (5, 18). A role for Sp1 in the phorbol ester induced differentiation dependent expression of human TGH in the macrophage THP-1 cell line, has been demonstrated (28). Overall, the rat TGH promoter shares 59% sequence identity with the murine TGH promoter sequence and 46% identity with the human TGH promoter. This appears to suggest that similar mechanisms for activation of TGH promoter activity operate in different species. Indeed previous evidence for sterol regulation of TGH expression and promoter activity in the rat and human has been presented (5, 18, 29), suggesting that the SRE-like elements are functional.

The TGH gene appeared to be an excellent candidate for targeted deletion. TGH is not significantly expressed in the mouse until after weaning (22, 23), therefore one would not expect an embryonic lethal phenotype. The present strategy involves the

elimination of the first exon containing the transcription and translation start sites as well as the proximal promoter of the murine TGH gene. Though this strategy will specifically eliminate TGH expression and allow us to investigate the function of the murine TGH protein by analyzing the phenotype of knock-out mice, one might expect the presence of three additional carboxylesterase genes on chromosome 8 to sufficiently compensate for the missing TGH. As we have presented in Fig. 3.3, CES1, LOC234564 and Es22 share a similar catalytic triad, secondary structure features and ER retrieval sequences that one might expect to retain these proteins within the ER lumen where TGH is also located. It has already been demonstrated that CES1 and Es22 are highly expressed in the liver (14, 15). At the moment little is known regarding the expression profile of the protein encoded by the LOC234564 gene. At the moment, there is no evidence that any of these three carboxylesterases hydrolyze lipid substrates or mobilize TG stored within the liver for secretion. Also, little is known regarding the regulation of the individual carboxylesterase genes. In the future, a strategy involving the elimination of these four genes (a deletion of approximately 170.4 kb) may be necessary to unmask a phenotype whereby the ability of the liver to mobilize stored TG for secretion is compromised.

In summary, the following evidence suggests that we have isolated the murine TGH gene: 1) the exon sequences match with the sequence of the murine TGH cDNA (16); 2) all splice acceptor and donor sequences match the consensus sequence of the splice junctions (7); and 3) the DNA sequence immediately upstream of the transcriptional start site contains a functional promoter (22).

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CHAPTER 4

THE REGULATION OF HEPATIC TRIACYLGLYCEROL HYDROLASE AND TRIACYLGLYCEROL LIPOLYSIS AND RE-ESTERIFICATION BY DEXAMETHASONE

4.1 Introduction

The liver secretes triacylglycerols (TG) as very low density lipoprotein (VLDL). The majority of the TG secreted by primary hepatocytes is derived from a cytosolic storage pool (1, 2). Stored TG undergoes an initial lipolysis followed by re-esterification of the lipolytic products to TG prior to incorporation into a VLDL particle (3-5). A triacylglycerol hydrolase (TGH), initially purified from porcine liver microsomes, has been implicated in this process (6). We cloned rat and mouse TGH cDNAs and discovered that TGH was a member of the carboxylesterase gene family (7, 8). Carboxylesterases contain the catalytic serine GXSXG and share the α/β hydrolase fold in common with lipases (9, 10). TGH is highly expressed in the liver (8, 11) and TGH accounts for approximately 70% of rat hepatic alkaline lipase activity (6, 12). TGH has been localized to regions of the ER in close contact with the lipid droplet (11). TGH is absent from hepatoma cell lines that secrete poorly lipidated VLDL (7). Transfection of McArdle RH7777 hepatoma cells with the rat TGH cDNA resulted in an increased mobilization of intracellular TG and lipidation of apoB100 (7). Furthermore, chemical inhibition of TGH reduced TG and apoB100 secretion by primary rat hepatocytes (12). Collectively, these data demonstrated that TGH is a lipase involved in the mobilization of stored TG some of which is secreted by the liver.

Following the lipolysis of stored TG, lipolytic products are re-synthesized to form TG at the ER where the TG may be utilized for lipoprotein assembly (13, 14). The final stage of TG synthesis is

catalyzed by diacylglycerol acyltransferase (DGAT). To date, two cDNAs encoding proteins with DGAT activity have been identified. DGAT1 is expressed in a wide variety of tissues, but targeted deletion of the murine DGAT1 gene failed to affect the levels of circulating TGs (15, 16). DGAT2 is most highly expressed in liver and adipose tissues (17).

The mechanism of VLDL assembly is complex and the potential for its regulation exists at a variety of levels. Since the rate of VLDL assembly and secretion depends upon the efficiency of apoB100 lipidation (18), hepatic TG lipolysis and re-esterification within the ER represents an important regulatory stage that exerts control over the lipidation of apoB100. At present, little is known regarding the regulation of the lipases and acyltransferases that catalyze the lipolysis and re-synthesis of TG in the liver. We recently cloned the murine TGH promoter and characterized the regulation of TGH at the time of weaning (19). The Sp1 transcription factor was identified as a positive regulatory factor that stimulated TGH expression at the time of weaning (19), a stage when the liver secretes larger quantities of TG (20).

The present experiments attempted to address whether or not stimulating TG biosynthesis altered hepatic lipolysis and re-esterification and the provision of stored TG for lipoprotein secretion. Glucocorticoids modulate lipid biosynthesis in the liver. For example, excess glucocorticoids are associated with increased *de novo* hepatic fatty acid and TG synthesis and increased circulating TG levels (21-27). Removal of glucocorticoids by adrenalectomy attenuated the development of obesity in a variety of genetic and dietary models of obesity (28, 29). Therefore, we

investigated whether TGH and the DGAT isoforms could be regulated by a synthetic glucocorticoid, dexamethasone, and whether intracellular TG turnover was affected.

4.2 Materials and Methods

4.2.1 *Reagents*

Restriction endonucleases, modifying enzymes, random primer labeling kit, Dulbecco's modified Eagle's medium (DMEM), sodium pyruvate, penicillin/streptomycin, fetal bovine and horse serum were obtained from Life Technologies Inc. Radioisotopes ([α-^{32}P]-dCTP, [γ-^{32}P]-dATP, [^{3}H]-glycerol, [^{14}C]-oleic acid) were from Amersham Biotech. Western blotting reagents were obtained from Amersham Canada. TaKaRa Ex-Taq high fidelity polymerase was from Takara Biomedicals Ltd. The Topo-TA cloning kit was from Invitrogen. Silica gel G plates were from Merck. Essentially fatty acid-free bovine serum albumin and all other chemicals were purchased from Sigma chemicals.

4.2.2 *PCR Primer Sequences*

All primers were synthesized at the DNA core facility, University of Alberta using a 394 DNA/RNA synthesizer (Applied Biosystems).

CYC1A: 5'-TCCAAAGACAGCAGAAAACTTTCG-3'; CYC2B: 5'-TCTTCTTGCTGGTCTTGCCATTCC -3'; DGAT1F: 5'-ATTCACGGATCATTGAGCG-3'; DGAT1R: 5'-CTGCCATGTCTGAGCATAGG-3'; DGAT2A: 5'- CTACGTTGGCTGGTAACTTCC-3'; DGAT2B: 5'-AACCAGATCAGCTCCATGG-3'; ESXF: 5'- CTATTCTTCCATGATGTGGCTCTGTG-3'; ESXR: 5'-CAAACATGACTGGGCCTCCTG-3'; ES22F: 5'-CCTGTAGCCTCCTACCATGTGC-3'; ES22R: 5'-GGGTGAGGCTGACAGAGTC-3'; Ex6F: 5'-CACTGCTGCTCTGATTACAAC

AG-3'; Ex10R: 5'-GCCTTCAGCGAGTGGATAGC-3'; 22.6F: 5'-CAGGCCTGGTCAAGAAGAACA -3'; 22.11R 5'- CAATTGCATCCT CAGGGAGG -3'; X7F: 5'- CTTGCCTTTTTGGGAGAGCTG -3'; X10R: 5'- CTGCCACAGGATGGATGCAG -3'; P-TGHII: 5'-GAGCAAAGTTGGCCCAGTATTTCATCACCATTTTGCTGAG-3'

4.2.3 *Cloning of murine DGAT2 and carboxylesterase cDNAs*

PCR amplification of murine TGH gene introns were carried out as follows: DGAT2 (GenBank Accession No. NM026384) primers DGAT2F-DGAT2R, 95°C 30s., 57°C 1 min., 72°C 1 min. for 30 cycles. Es 22 (GenBank Accession No. S80191) primers ES22F-ES22R, 95°C 1 min., 57°C 1 min., 72°C 3 min. for 35 cycles. Es X (GenBank Accession No. NM021456) primers ESXF- ESXR, 95°C 1 min., 58°C 1 min., 72°C 3 min. for 35 cycles. PCR products were cloned into pCR2.1-Topo (Invitrogen), following the manufacturer's protocol. Cloned murine cDNAs were sequenced as described previously and confirmed by comparison to the sequences available in the GenBank database.

4.2.4 *Reverse transcription of RNA and quantitation of mRNA expression by real-time PCR*

Total RNA was reverse transcribed using an oligo dT_{20} primer and Superscript II reverse transcriptase (Invitrogen) according to the manufacter's instructions. TGH (primers EX6F-EX10R), Es 22 (primers 22.6F-22.11R), EsX (primers X7F-X10R), DGAT1 (primers DGAT1F-DGAT1R), DGAT2 (primers DGAT2A-DGAT2B) and cyclophilin (primers CYC1A-CYC2B) transcripts were detected by real time PCR using a LightCycler (Roche Diagnostics) machine.

Reaction mixtures contained 0.5mM dNTPs, 3mM $MgCl_2$, 2.5μM of each primer, 1X SYBR Green I (Molecular Probes) in DMSO and 3U Taq polymerase in a total volume of 20 μl. Amplification was carried out as follows: 95°C, 57°C 10 s., 72°C 15 s. The number of cycles is indicated in the figure legends. Data analyses was performed using the LightCycler Software version 3.5 (Roche).

4.2.5 *Maintenance of animals*

Male C57/BL6 mice (6 weeks of age) were housed in a thermostatically controlled room with artificial lighting (12 hours light/12 hours dark). Animals had unrestricted access to water and to a commercial pelleted diet (22% protein/22% fat/56% carbohydrate). Mice were injected intraperitoneally with 40 mg/kg body weight dexamethasone (dex) delivered in 200 μl of corn oil, for 4 consecutive days and sacrificed 24h after the final injection, as described in a previous study (31). Control mice received corn oil only.

4.2.6 *RNA isolation and Northern analysis*

Total murine tissue RNA was isolated using Trizol Reagent (Life Technologies) according to the manufacturer's instructions. To perform Northern blot analysis, total RNA was separated in a 1% agarose/2.2M formaldehyde gel and transferred to a Hybond-N+ nylon membrane (Amersham) using a vacuum blotter (Biorad) according to manufacturer's instructions. Pre-hybridization and hybridization steps were carried out in 0.144M $NaHPO_4$, 7% SDS, 2μM $Na_4P_2O_7$, 2μM EDTA, 100μg ml^{-1} sheared salmon testes DNA

(Sigma) and 1.5 x 10^6 cpm ml^{-1} at 55°C. For detection of TGH, ^{32}P-labelled 40bp oligonucleotide P-TGHII was utilized. The full length ^{32}P-labelled DGAT1 (kind gift of Dr. R.V. Farese), DGAT2 and PDI (kind gift of Dr. M. Michalak) cDNAs were utilized for their respective detection by Northern analysis.

4.2.7 *Isolation of crude microsomal fractions and protein determination*

Crude microsomal fractions were prepared for assay of enzyme activities and immunoblotting. Mouse livers were isolated and rinsed in ice cold PBS. Subsequently, a 10% homogenate was prepared by homogenizing the liver (Polytron 20 s burst) in homogenization buffer containing 10mM Tris-HCl, 0.25M sucrose, 2mM EDTA, pH 7.4. The homogenate was centrifuged at 1000X g for 20 min. The resulting pellet was discarded and the supernatant was centrifuged at 12 000 X g for 10 min. The resulting pellet was resuspended in 5 ml of homogenizing buffer by 15 strokes of a loose fitting, hand held dounce tissue homogenizer and again centrifuged at 12 000 X g for 10 min. The resulting pellet was discarded (the mitochondrial fraction) and the post-mitochondrial supernatant was centrifuged for 60 min at 100 000 X g. The resulting pellet was resuspended in 0.5 ml of homogenizing buffer by 15 strokes of a tight fitting, hand held dounce tissue homogenizer and used as the source of the microsomal fraction. The microsomal fraction was aliquoted into 1.5 ml microfuge tubes and frozen at -70 °C. Marker enzyme analyses revealed the microsomal fraction was contaminated with 5% mitochondria particles (32). The protein concentration of the crude microsomal fraction was determined by a

BCA protein assay (Pierce) (33).

4.2.8 *Immunoblot analysis*

Mouse liver microsomes (25μg of protein) were electrophoresed on a SDS/10% (w/v) polyacrylamide gel, transferred to a nitrocellulose membrane, and the expression of TGH was analyzed by blotting with the anti-TGH polyclonal antibody as described previously (12). The expression of PDI was analyzed by blotting with the anti-PDI polyclonal antibody (Santa Cruz) according to the manufacturer's instructions.

4.2.9 *Lipase assay*

Lipolytic activity in murine liver microsomes were assessed using the fluorogenic substrate, 4-methylumbelliferyl heptanoate (MUH). A 1mM stock of MUH was prepared in tetrahydrofuran. The enzymatic reaction begins with the injection of 2.5μM MUH in 20mM Tris buffer pH 8.0, 1mM EDTA, 300μM taurodeoxycholate to 1μg of microsomal protein in a 96 well plate (total volume 100μl). The plate is agitated at room temperature and read with a Fluoroskan Ascent FL Type 374 (Thermo Labsystems) in a kinetic fashion up to 10 min (excitation/emission wavelengths: 355/460nm). Data was analyzed using Ascent software version 2.4.2.

4.2.10 *Diacylglycerol acyltransferase assay*

The synthesis of TG from sn-1,2(2,3)[^{3}H]-dioleoylglycerol was measured in crude liver microsomes as described previously (34). The assay mixture contains 20mM Tris-HCl, pH 7.4, 150mM sodium

chloride, 4mM magnesium chloride, 20mM sodium fluoride, 1mM DTT and 0.1% CHAPS. A substrate suspension of 25μM DG (50 000 dpm/assay) and 50μM oleoyl-CoA was prepared by sonication in the assay mixture. The reaction begins with the addition of 50μg of mouse liver microsomes to the assay mixture (final volume 200μl). The reaction proceeds for 10 min at 37°C and is terminated by the addition of 4ml of chloroform:methanol (2:1) and 750 μl of water. Samples were centrifuged at 2000 rpm for 10 min to separate organic and aqueous phases. The aqueous phase was removed and the organic phase dried under nitrogen. Lipids were resuspended in 50μl of chloroform:methanol (2:1) and spotted on a TLC plate. Lipids were separated in hexane:diethyl ether:acetic acid (80:20:1) solvent and visualized by staining in iodine vapor. Radioactive TG was determined by scintillation counting.

4.2.12 *Gas chromatographic determination of TG mass*

In order to analyze TG mass by gas chromatography, 200μl of plasma and 2ml cell media, cells/tissues are digested with 2 units of phospholipase C from *Clostridium welchii* (Sigma) to remove polar head groups. Free hydroxyl residues are converted to TMS ether/ester derivatives. Samples were separated using an Agilent 6890 Series gas chromatograph (Agilent Technologies) using an HP-5 column. Data analyses were performed using GC Chemstation software (Agilent Technologies).

4.2.13 *Hepatic TG Secretion Rate*

Mice were injected intraperitoneally 4 times daily for four days with

40mg/kg dex (controls received vehicle only), as described in 4.2.5. 24h following the final injection, and following an overnight fast, a blood sample was removed (baseline) and then mice were injected with 20 mg Triton WR-1339 in a total volume of 100μl (46). Subsequently, a 50μl blood sample was removed from the leg vein 1h and 2h following the Triton injection. Plasma TG concentrations were determined by enzymatic methods using the GPO-Trinder kit (Sigma).

4.2.14 *Preparation of hepatocyte cultures from mice*

Hepatocytes were prepared under sterile conditions from mice (17-24g body weight) at 10 AM (approximately 6 h into the light phase of the cycle), by collagenase perfusion of the liver. Hepatocytes were suspended at a concentration of 1.5 X 10^6 cells/ml in DMEM containing fetal bovine serum (10% v/v), penicillin (15μg/ml) and streptomycin (15U/ml). The cell suspension (2 ml) was plated on 60mm collagen coated dishes and the cells were permitted to form a monolayer (2 h). Following cell attachment, the medium was removed and cells were washed twice with phosphate buffered saline (PBS) and cells were further cultured in DMEM containing the above antibiotics in the absence of serum.

4.2.15 *Pulse chase experiments with primary mouse hepatocytes*

Cells were prepared as decribed above from either a control mouse or a mouse that received 40 mg/kg body weight dex. Following cell attachment, the medium was replaced by DMEM containing [^{3}H] glycerol (0.25mM, 10μCi), [^{14}C] oleate (0.75mM, 1μCi) and 0.5%

bovine serum albumin in order to pre-label the cellular TG stores. After 4 h, media was removed and cells were washed twice with PBS. Some dishes were harvested (Pulse) whereas others were cultured in DMEM in the absence of exogenous glycerol and oleate for a further 18 h (Chase). After 18 h, media and cellular lipids were isolated and analyzed.

4.2.16 *Lipid extraction and analysis*

After washing the cell monolayer with 1ml cold PBS, 1 ml of cold PBS was added to each dish and cells were scraped from the dish with a rubber policeman and transferred to a glass tube. A 50μl aliquot was removed for determination of protein. Addition of 6 ml of chloroform:methanol (2:1) and 1 ml of 0.73% NaCl to the tube facilitated separation of organic and aqueous phases. Tubes were centrifuged at 2000 rpm for 10 min and the upper (aqueous) phase was removed. The remaining organic phase was washed with 5 ml of theoretical upper phase (chloroform:methanol:0.9% NaCl 3:48:47 v/v). Tubes were again centrifuged at 2000 rpm for 10 min and the upper phase was removed. The lower (organic) phase was dried under nitrogen. The lipid sample was redissolved in 50μl of chloroform:methanol (2:1) and applied to a TLC plate. Lipids were separated using the chloroform:methanol:acetic acid:water (75:45:12:6) solvent system, allowing the solvent to proceed up to one third of the plate. The TLC plate was dried and further separation of neutral lipids was achieved in the heptane:isopropyl ether:acetic acid (90:60:6) solvent system, allowing the solvent to proceed up the entire height of the plate. Lipids were visualized by

iodine vapor and radioactive TG determined by scintillation counting.

4.2.17 *Calculation of intracellular TG turnover*

Culturing cells in which the intracellular TG has been doubly labeled with [^{3}H] glycerol and [^{14}C] oleate gives rise to a relative decline in the specific radioactivity of the [^{3}H] label compared to that of the [^{14}C] label (35). The glycerol and fatty acid moieties appeared to undergo differential metabolism in the absence of exogenously supplied oleate and glycerol. The extent of the dilution of the TG-glycerol pool reflects the degree of TG lipolysis and re-esterification of the [^{14}C]-oleate with a [^{3}H]-glycerol pool of a lower specific radioactivity. Therefore, we define the initial (Pulse) [^{3}H]:[^{14}C] specific activity ratio of the cellular TG as X and the final specific activity ratio of TG as Y. If X = Y then no excess glycerol label has entered the TG pool and lipolysis is zero. If X > Y then excess unlabeled glycerol has entered the pool. This may be represented as the fractional turnover of the cellular TG pool ($^{X}/_{Y}$ - 1). Because the total (cellular and media) TG pool size at the end of the culture period is known, the absolute TG turnover may be calculated as the product of the fractional turnover multiplied by the TG pool size.

4.2.18 *PCR-Based Nuclear Run-on Assay*

This assay was performed essentially as described (36). Briefly, highly purified and transcriptionally active nuclei were prepared from murine liver according to the methods of Marzluff and Huang (37). Freshly prepared or frozen/thawed nuclei (200 µl) were split into two

aliquots and incubated for 30 min at 30°C in 20% glycerol, 30mM Tris-HCl (pH 8.0), 2.5mM $MgCl_2$, 150mM KCl, 1mM DTT and 40U of RNasin (Promega) with or without 0.5 mM (each) ribonucleoside triphosphates (+/- rNTPs). After 30 min, nuclei were lysed by the addition of 200 µl of 4M guanidinium thiocyanate, 25mM sodium citrate (pH 4.0), 0.5% sarcosyl, 0.1M 2-mercaptoethanol. Yeast tRNA (20 µg) was added and RNA was extracted by the acid-guanidinium-thiocyanate method then resuspended in water treated with diethylpyrocarbonate. RT-PCR was performed as described in 4.2.4. Because the run-on products are not labeled, newly transcribed mRNA in isolated nuclei was detected following nuclear transcription initiated by the addition of exogenous rNTPs (+ rNTPs). Nuclear transcription reactions lacking exogenous rNTPs (- rNTPs) were included to control for mRNAs synthesized by endogenous rNTPs in isolated nuclei.

4.2.19 *Transfections and Reporter Assays*

Rat McArdle RH7777 cells (CRL-1601 American Type Culture Collection) were plated and transiently transfected with TGH promoter luciferase reporter constructs (5µg) using a cationic liposome technique. All plates received 2µg of pSV-βgal (Promega) as a control for transfection efficiency. Deletion mutants of the murine TGH promoter were previously created, as described (19). Reporter assays using cell lysates were performed as recommended (Promega) and luminometric measurements were made using a Fluoroskan Ascent FL Type 374 (Thermo Labsystems). Luciferase activity was normalized to beta-galactosidase activity (Promega).

4.3 Results

4.3.1 *Dexamethasone treatment increases TG in the liver and plasma.*

Excess glucocorticoids are associated with reduced body weight gain and increased plasma and hepatic TG (38-40). We injected mice with 40 mg/kg body weight dex for 4 days. Compared to the controls, the dex-injected mice had a lower weight on average (Table 4.1). Excess glucocorticoids are also associated with an increased liver weight and an increased liver/body weight ratio. We have observed both a higher liver and liver/body weight ratio in the mice that were injected with dex compared to controls (Table 4.1). Hepatic TG and plasma TG accumulation in hypercortisolemic humans and rodents has been observed (25, 26, 28). In the dex-injected mice, we have observed a 5-fold ($p<0.001$) increase of hepatic TGs (Table 4.1). In addition, the plasma TG concentration, measured after an overnight fast (therefore excluding chylomicron TGs), was also observed to be 2.2-fold ($p<0.001$) higher in the dex-injected mice than the control mice (Table 4.2). Taken together, this model of the dex-injected mouse exhibits metabolic alterations associated with excess glucocorticoids.

Table 4.1- Body and liver characteristics of mice used in study.

	Control		Dex		*t*-test
Body Weight (g)	20.57 ±	1.68	18.86 ±	1.18	0.051
Liver Weight (g)	0.284 ±	0.034	0.322 ±	0.046	0.053
Liver/Body Weight Ratio	1.377 ±	0.074	1.789 ±	0.187	0.001
Liver TG (μg/mg protein) n=6	212 ±	97	1 095 ±	291	0.001
Total Hepatocyte Cell TG (μg/mg protein) n=15	329.1 ±	121.3	620.95 ±	180.7	0.026

Table 4.2- Hepatic TG secretion and plasma TG levels.

	Control		Dex		*t*-test
Plasma TG (mg/dL) n=9	30.49 ±	12.82	67.82 ±	26.39	0.0003
Hepatic TG Secretion Rate (mg/kg body weight/h) n=10	83.89 ±	8.35	91.81 ±	16.76	0.190
Total Hepatocyte Media TG (μg/mg protein) n=15	50.87 ±	16.0	38.33 ±	15.8	0.284

4.3.2 *Dexamethasone reduces TGH mRNA expression and increases DGAT mRNA expression in mouse liver.*

The mRNA expression of several genes in the livers of control and dex injected mice was examined (Fig. 4.1). The expression of DGAT1 was increased ~1.6-fold and DGAT2 was increased ~1.7-fold relative to protein disulfide isomerase (PDI) in the livers of dex-injected mice, compared with controls (Fig. 4.2). TGH mRNA was significantly decreased ~40% relative to PDI by dex (Fig. 4.2).

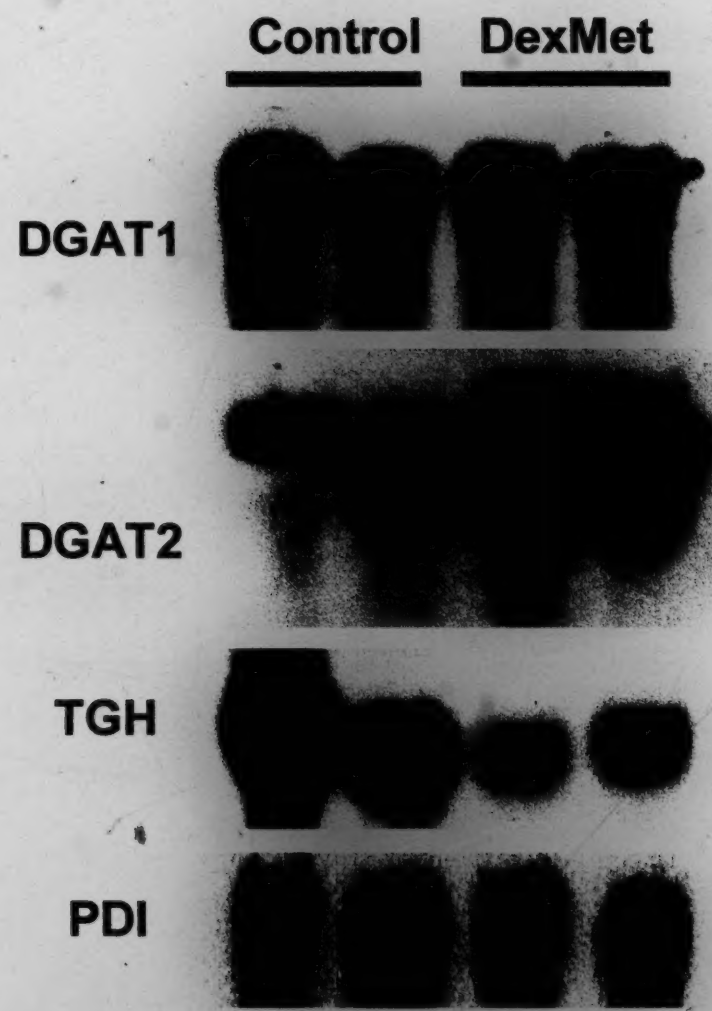

Fig. 4.1- Dexamethasone regulates the mRNA expression of the enzymes of lipolysis and re-esterification in the liver. Northern blotting was performed as described in methods. Murine TGH mRNA was detected by hybridization with a ^{32}P labeled TGH-specific oligonucleotide probe. Mouse DGAT1, DGAT2 and PDI mRNAs were detected with their respective ^{32}P labeled cDNA probes.

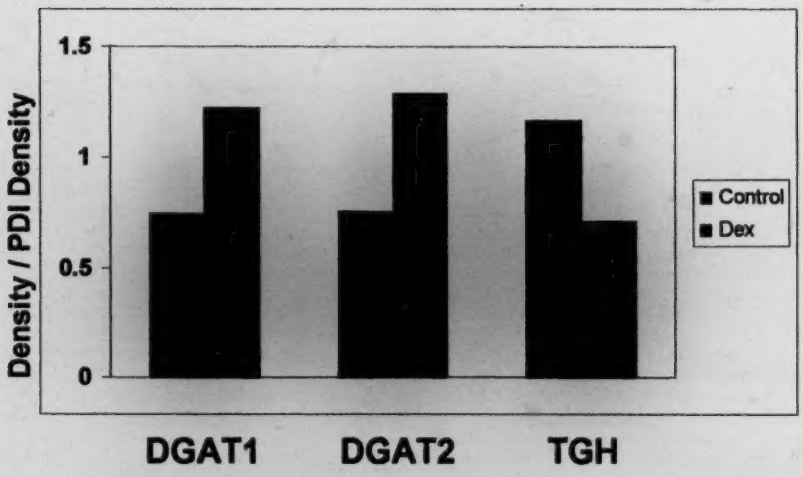

Fig. 4.2- Northern blot densitometry of messages expressed relative to the PDI message..

4.3.3 *Dexamethasone increases in vitro microsomal diacylglycerol acyltransferase activity in mouse liver microsomes.*

Though evidence shows that TG biosynthesis is stimulated by dex, a possible stimulation of hepatic DGAT activity by dex has not been reported previously. Since the level of DGAT1 mRNA and protein expression do not necessarily correlate with DGAT activity (41, 42), microsomal DGAT activity was measured using [^{3}H]-DG as the substrate. DGAT activity was increased 1.2-fold (P=0.02) in murine hepatic microsomes from dex- compared to vehicle-injected mice (Fig. 4.3). Since DGAT-isoform specific antibodies are not available, we could not investigate whether or not the changes in mRNA and activity correlated with microsomal protein content. However, our assay conditions (low [Mg^{++}] ensures the measurements of both DGAT1 and DGAT2 activities simultaneously.

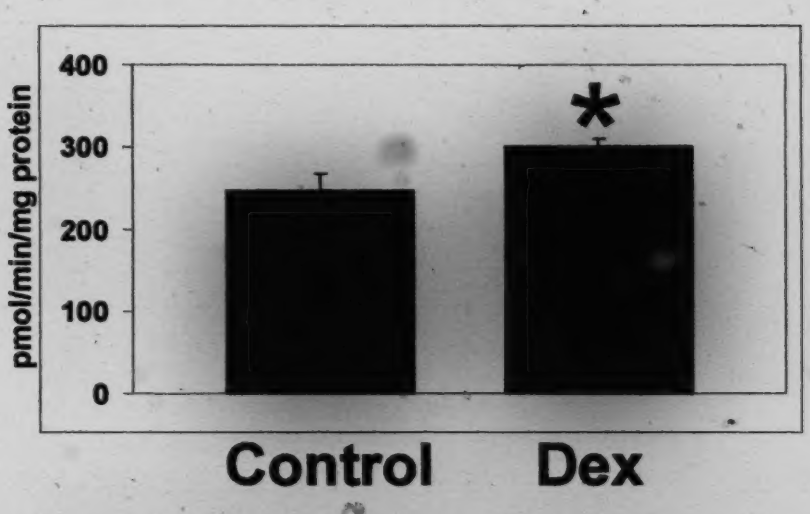

Fig. 4.3- Dexamethasone increases the diacylglycerol acyltransferase (DGAT) activity 1.2-fold in murine liver microsomes. Liver microsomes were prepared from murine livers harvested from control and dex-injected mice. *In vitro* DGAT activity was measured by the synthesis of triacylglycerol from [^{3}H]-DG as described in methods. n=3, *p =0.02 one experiment.

4.3.4 *Dexamethasone reduces microsomal esterase activity in mouse microsomes.*

Hydrolysis of the fluorogenic substrate, MUH, was measured using crude microsomal preparations. Microsomal esterase activity was reduced by approximately 40% ($P<0.005$) in microsomes from dex injected mice, compared to controls (Fig. 4.4). Since a polyclonal anti-TGH antibody is available, we showed that TGH protein expression was reduced 40% in hepatic microsomes from dex-injected mice compared to mice that received vehicle alone (Fig. 4.5). This result correlates with the decreased esterase activity and the results of others that have demonstrated that rat liver microsomal carboxylesterase activity was regulated by dex (31, 43). Since TGH accounts for the majority of microsomal esterase activity in the liver (12), the reduced esterase activity in murine hepatic microsomes from dex injected mice was primarily a consequence of the reduced expression of TGH in their livers.

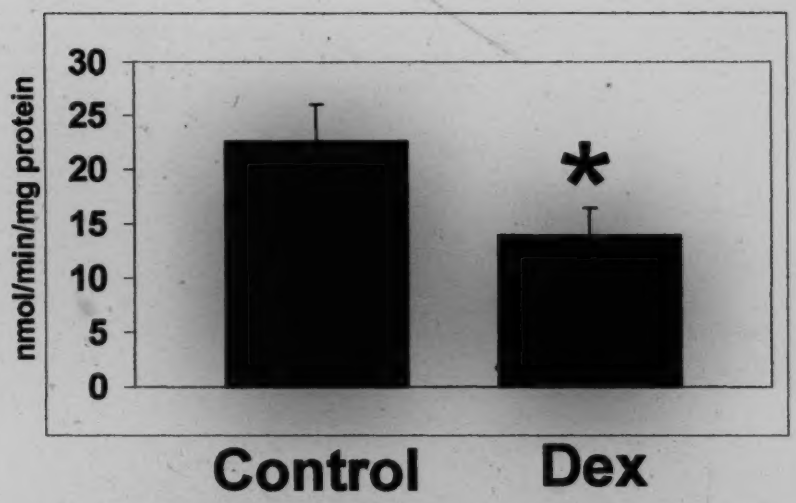

Fig. 4.4- Dexamethasone reduces the murine hepatic microsomal esterase activity 40%. Microsomes were prepared from livers harvested from control and dex-injected mice. *In vitro* esterase activity was measured by the hydrolysis of 5-methylumbelliferylheptanoate as desribed in methods. The extent of hydrolysis was determined by measuring the excitation at 355nm and emission at 460nm in a kinetic fashion. n=3, *p =0.005.

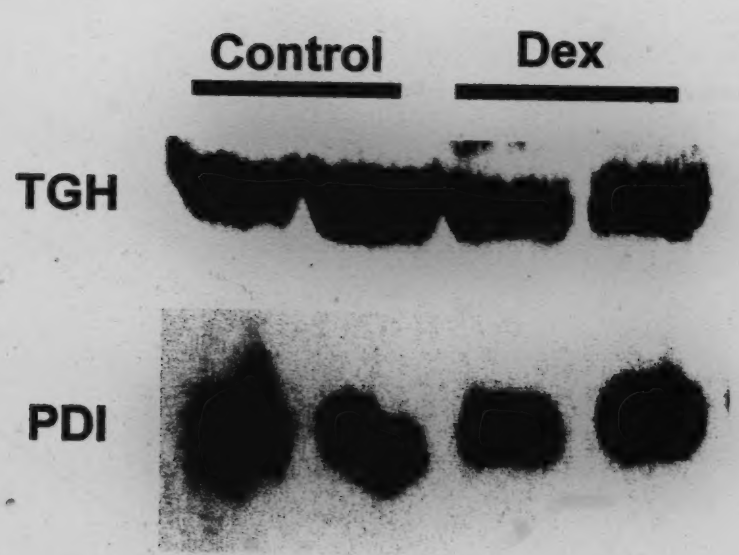

Fig. 4.5- Dexamethasone reduces TGH protein expression in murine hepatic microsomes. Western blotting was performed as described in methods. TGH and PDI proteins were detected using their respective anti-TGH and anti-PDI polyclonal antibodies.

4.3.5 *Validation of RT-PCR assay of carboxylesterases*

As discussed in Chapters 2 and 3, TGH is a member of the carboxylesterase gene family. To measure the mRNA expression of individual carboxylesterases, an RT-PCR assay was designed that could accurately distinguish between TGH and the most closely related murine carboxylesterases, Es22 (44) and EsX (45). The ability of the specific primer set to amplify a target sequence was determined utilizing 20 ng of the isolated cDNA sequence and reverse transcribed liver mRNA. TGH specific primers amplified the target sequence only from the murine TGH cDNA and a hepatic cDNA library and not murine Es22 or EsX cDNAs (Fig. 4.6A). Amplification of TGH was confirmed by sequencing the PCR product. In addition, Es22 and EsX specific primers only amplified their respective cDNAs (Fig. 4.6B, C). All primer sets amplified a target sequence from reverse transcribed liver mRNA.

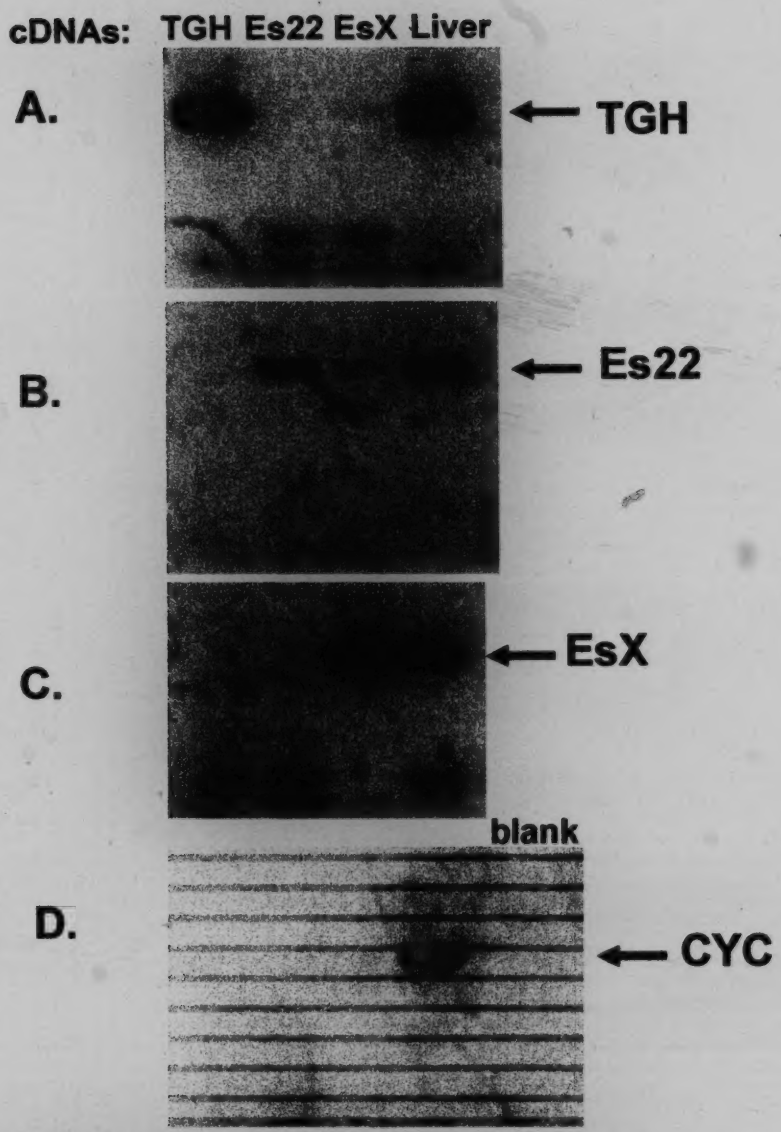

Fig. 4.6- Positive control for specificity of RT-PCR analysis of carboxylesterase mRNA expression. The ability of primers specific for A. TGH; B. Es22; C. EsX; D. cyclophilin (CYC); to amplify their target sequences (35 cycles) from either the TGH, Es22, EsX cDNAs or a murine liver mRNA library.

4.3.6 *Characterization of the expression of the mRNAs for TGH, DGAT1 and DGAT2 in the primary murine hepatocyte model.*

The primary hepatocyte model is a well characterized model for measuring the contribution of the lipolysis and re-esterification pathway to TG secretion (3, 4). First, we determined by RT-PCR whether or not cultured primary murine hepatocytes derived from control animals express mRNAs for enzymes involved in TG lipolysis and re-esterification and followed the length of time in culture that hepatocytes maintained the expression of these mRNAs (Fig. 4.7). DGAT1 mRNA expression could be detected up to 72h in cell culture, but declined after 36h. DGAT2 mRNA expression could be detected in hepatocytes up to 42h in culture, but DGAT2 expression markedly declined after 24h in culture. TGH mRNA is expressed for 72h, but its expression declines after 48h. As a further control, the expression of the mRNAs for DGAT1, DGAT2 and TGH in primary murine hepatocytes derived from control or a dex-injected mouse was examined after 24h in culture (Fig. 4.8). As is the case for murine liver, the mRNAs for DGAT1 and DGAT2 expression were higher in hepatocytes prepared from dex-injected mice following 24h of incubation compared to control cells (Fig. 4.8). TGH mRNA expression was significantly reduced in hepatocytes from dex-injected mice compared with controls, following 24h of incubation compared to control cells. Therefore changes in TGH as well as DGAT-1 and -2 expression induced by dex observed in the cultured primary mouse hepatocytes parallel those observed in the whole murine liver.

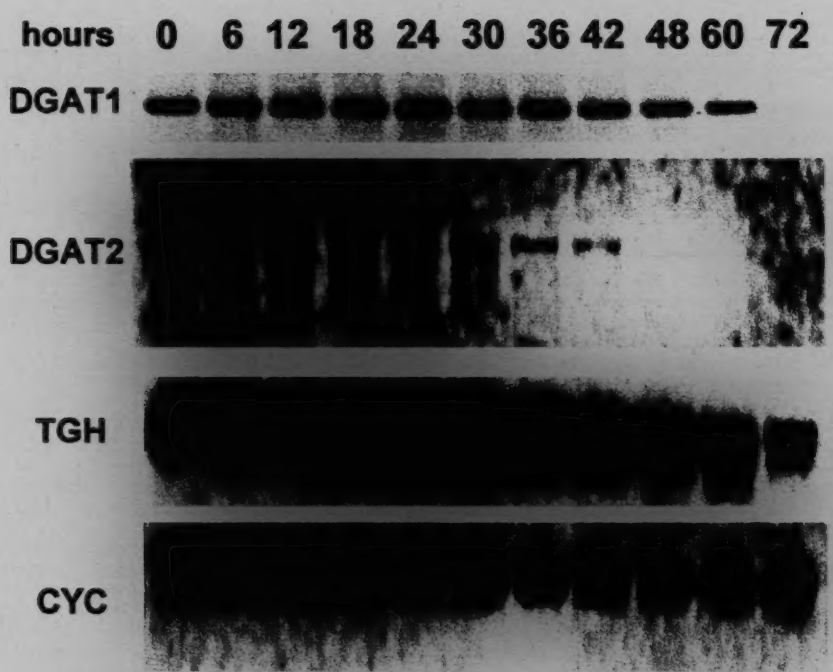

Fig. 4.7- Expression of the mRNA for the enzymes of TG lipolysis and re-esterification in hepatocyte cell cultures depends on the age of the culture. Cells derived from control mice were harvested following at the indicated times and RNA was extracted. DGAT1, DGAT2 and TGH mRNA expression was detected by RT-PCR (40 cycles).

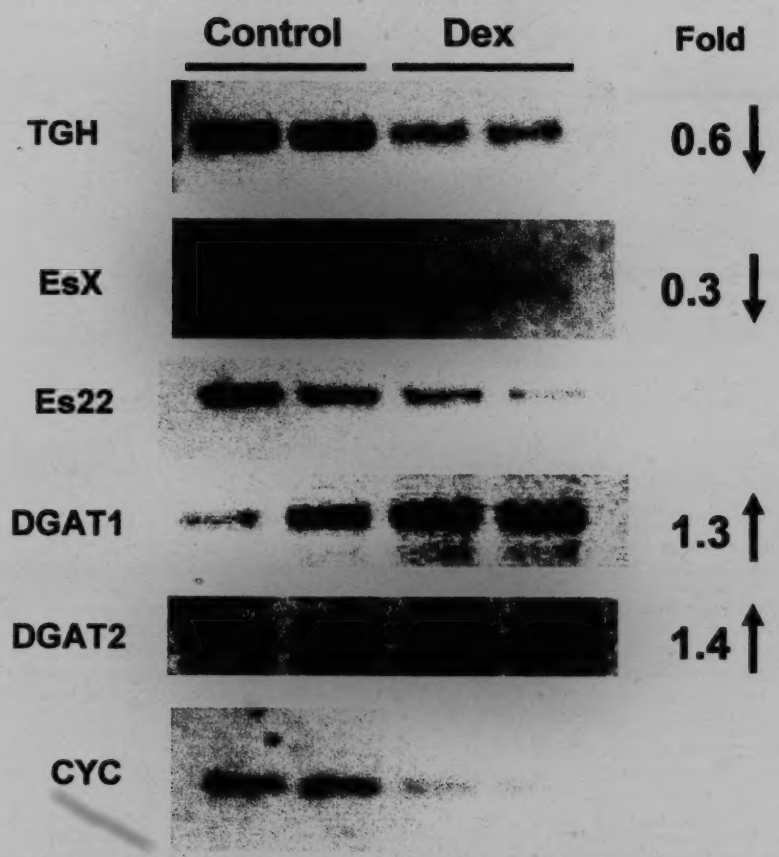

Fig. 4.8- Expression of the mRNAs for DGAT1 and 2 is higher and TGH is lower following 24h in hepatocyte cell cultures derived from dex-injected mice. Cells derived from control and dex-injected mice were harvested following a 24h incubation and RNA was extracted. DGAT1 and 2, TGH, Es22, EsX mRNA expression were determined by RT-PCR as described previously (22 cycles). Fold changes in mRNA expression are calculated relative to CYC.

4.3.7 *Intracellular TG turnover in primary murine hepatocytes.*

The stored TG within hepatocytes undergoes lipolysis followed by re-esterification prior to secretion (2-4). To assess whether dex regulated the intracellular turnover of stored TG, we utilized primary murine hepatocytes derived from control and dex-injected mice. A dual-labeling, pulse-chase experiment was designed (see 4.2.15) to calculate the intracellular TG turnover and secretion by murine hepatocytes (see 4.2.16), that falls within the 24h window in which TGH, DGAT1 and DGAT2 expression was maintained at a high level (Fig. 4.7). This experiment is similar to a previous study that examined TG turnover by rat hepatocytes (35). Figure 4.9 shows that murine hepatocytes derived from the dex injected mice turned over (lipolysis followed by re-esterification) 7.25μg TG/mg protein/h compared to 14.0μg TG/mg protein/h by the hepatocytes from control mice. This represents almost a 50% slower rate of turnover by the labeled TG in the storage pool of hepatocytes from dex mice during the 18h chase period compared to the hepatocytes from control mice.

4.3.8 *Dexamethasone does not alter the hepatic TG secretion rate in mice.*

The non-ionic detergent, Triton WR-1339 was used to determine rates of hepatic TG entry into the circulation (46). We observed no statistically significant differences in the rate of TG secretion between control and dex-injected mice (Table 4.2). Furthermore, when hepatocytes were cultured under the same conditions as the cells in the TG turnover study (see 4.2.15) no statistically significant differences were observed between the mass

of TG secreted into the media by murine hepatocytes derived from dex-injected mice compared to controls during an 18h period (Table 4.2).

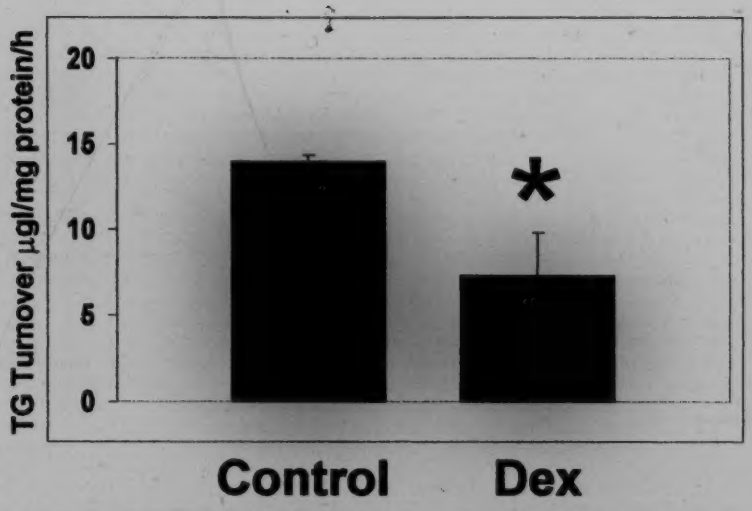

Fig. 4.9- Dexamethasone suppresses intracellular TG turnover. Hepatocytes were cultured as described in methods. After 4 h in the presence of [^{14}C]oleate (0.75 mM) and [^{3}H] glycerol (0.25 mM), cells from some dishes were harvested and the [^{3}H] and [^{14}C] specific activity of the cellular TG determined. The remaining dishes were cultured for an additional 18 hours in the absence of oleate and glycerol. At the end of this period, the[^{3}H], [^{14}C] and specific activity of TG was calculated as described in methods. Values represent the mean ± SD of 3 independent hepatocyte preparations (n=3) *P=0.01.

4.3.9 *Effects of dex on transcriptional regulation*

Both nuclear run on assays and TGH promoter-reporter gene transfection assays were performed to determine whether dex regulated TGH gene expression at the transcriptional level. Transcriptionally active nuclei were isolated from livers of dex-injected and control mice. As shown in Fig. 4.10, dex treatment had no effect on the run-on activity of the TGH gene compared to cyclophilin. To further verify this observation, transient transfection assays were carried out on dex-treated and control McArdle RH7777 rat hepatoma cells with three different murine TGH promoter-reporter gene plasmids (Fig. 4.11). The difference of activity between dex-treated and untreated cells was not significant. This suggested that transcriptional regulation was not involved in the dex-mediated TGH gene suppression. The activity of different deletion mutants was consistent with previous results (19).

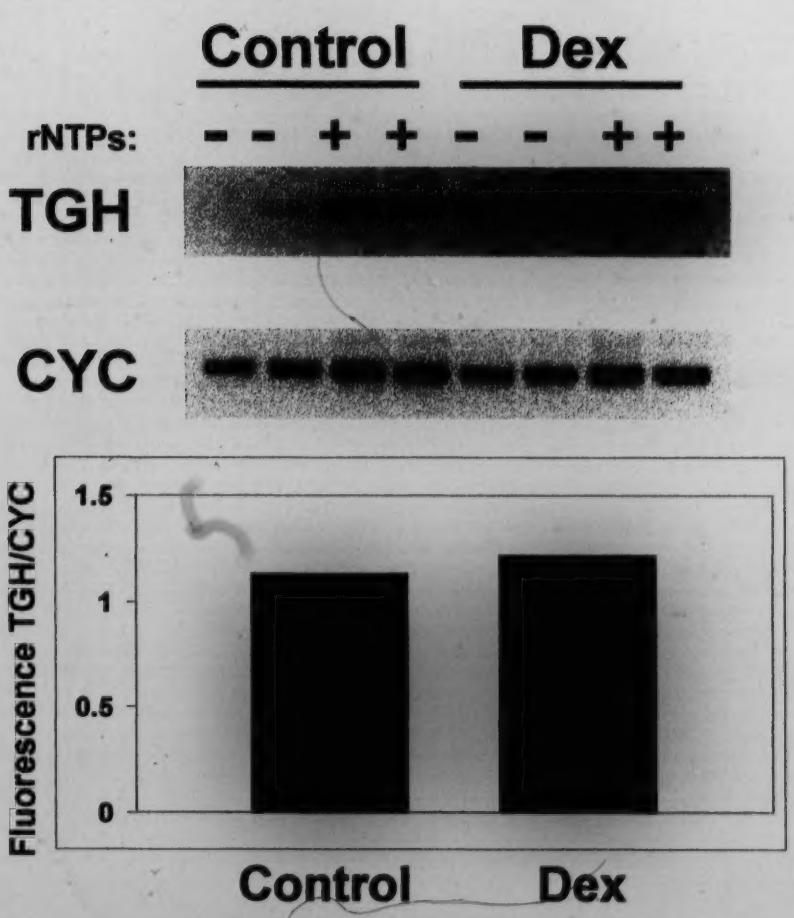

Fig. 4.10- Nuclear run-on assay of TGH transcription in liver nuclei isolated from dex-injected and control mice. Nuclei were isolated and used for nuclear run-on assays as described in methods. RT-PCR was used as described previously (25 cycles). Using cyclophilin (CYC) as a control, dex had no effect on TGH transcription. Gel is representative of two independent experiments.

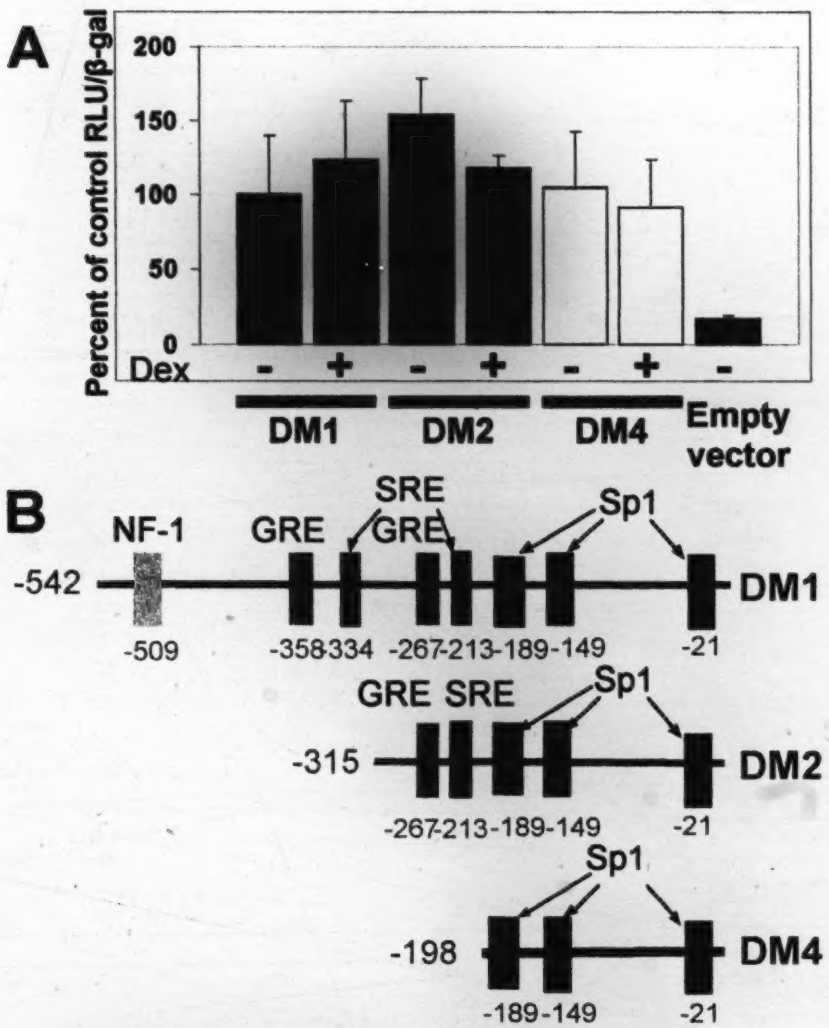

Fig. 4.11- Promoter activity of TGH-luciferase gene fusion constructs in McArdleRH7777 hepatoma cells.
A. Luciferase activity in transiently transfected McArdle RH7777 cells incubated 24h +/- 1μM dexamethasone, average of three independent experiments. **B.** Nested 5'-deletions of the murine TGH promoter upstream of the luciferase coding region in pGL3Basic and putative transcription factor binding sites are indicated, NF-1, nuclear factor 1, GRE, glucocorticoid response element half-sites, SRE, sterol response element.

4.3.10 *Effects of dex on mRNA stability*

As shown in Fig. 4.7, TGH expression was maintained at a high level over the long-term in murine hepatocyte cultures. Using actinomycin D to block new transcription, the half-life of TGH mRNA in primary mouse hepatocytes was examined and determined to be around 8 h, suggesting that TGH mRNA is very stable (Fig. 4.12A). As shown in Fig. 4.12B, treatment of hepatocytes with dex, caused a decrease in TGH mRNA by 4 h. This result suggested that dex decreases the stability of TGH mRNA in hepatocyte culture. However, simultaneous addition of actinomycin D increased the stability of TGH mRNA in the presence of dex (Fig. 4.12C). This result suggested that the reduction of TGH mRNA level by dex treatment was actinomycin D sensitive.

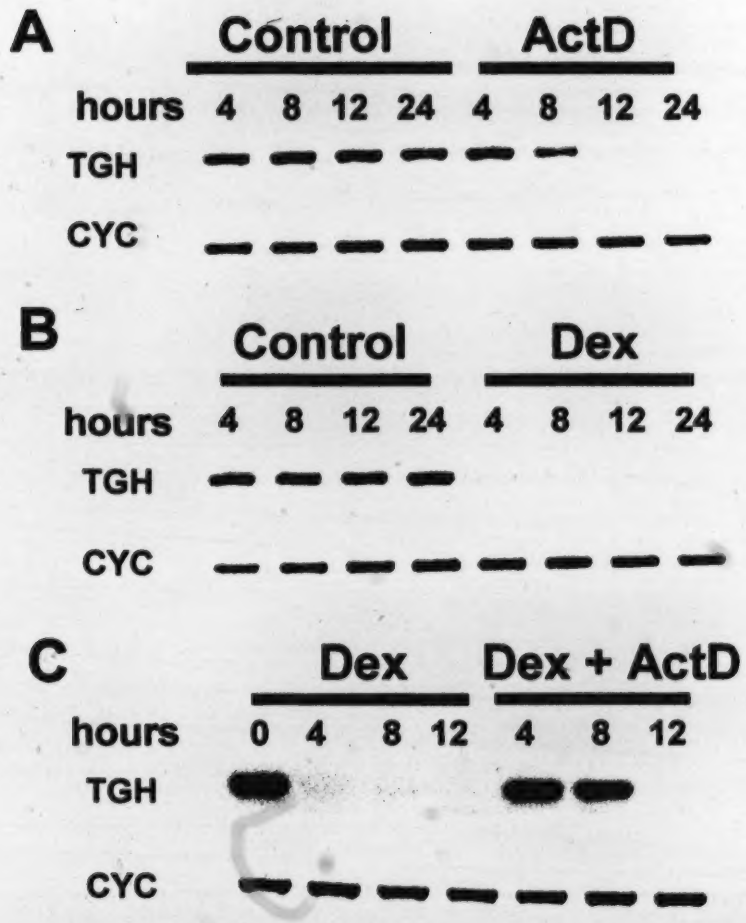

Fig. 4.12- Dexamethasone (Dex) regulates the stability of TGH mRNA in primary mouse hepatocytes. **A.** Cells were plated and incubated in the presence or absence of 10μg/ml ActD for the indicated time and expression determined by RT-PCR as described in 4.2.4. **B.** Cells were plated and incubated for the indicated times in the presence or absence of 1 μM Dex. **C.** Cells were plated and incubated for the indicated times in the presence of 1 μM Dex or a combination of 1 μM Dex and 1 μg/ml ActD for the indicated times. Representative gels of three independent hepatocyte preparations are shown.

1801 5′- UAUUAUUC -3′ 1807

1866 5′- UAUUUAAU -3′ 1873

1896 5′- UAUUUAUC -3′ 2002

Consensus:

5′- UAUUUA(U/A) (U/A) -3′

Fig. 4.13- Location of AU-rich elements in the 3'-untranslated sequence of the murine TGH mRNA.

4.4 Discussion

Glucocorticoids cause an increase in circulating TG and increased TG synthesis and storage (21-24, 27, 28). Dex is a potent analog of glucocorticoids (cortisol and corticosterone). Dex induces similar effects as natural glucocorticoids (27). The dex-injected mouse exhibits the physiologic and metabolic alterations in lipid metabolism associated with excess glucocorticoid. These included increased liver/body weight ratio, hepatic and plasma TGs (Table 4.1). The liver is central to lipid homeostasis and has a great capacity to store TG and secrete TG as apoB containing lipoprotein particles. The rate of VLDL-TG secretion is determined by the efficiency of apoB lipidation (18). Since dex does not affect the expression of apoB (27, 47) or the microsomal triglyceride transfer protein (48), we examined whether the changes in TG levels in the dex-injected mice could be associated with changes in the expression and activities of enzymes that catalyze hepatic TG lipolysis and esterification. We found that DGAT-1 and DGAT-2 expression was increased in the livers from mice that received dex-injections, compared to controls (Fig. 4.1, 2) whereas TGH mRNA and protein expression was decreased (Fig. 4.1, 4.2, 4.5). In agreement, hepatic microsomal DGAT activity was increased by ~1.2-fold (Fig. 4.3) and hepatic microsomal esterase activity, primarily a reflection of TGH activity (6, 12), was decreased by dex (Fig. 4.4). Next, we showed that in primary hepatocytes derived from dex-injected mice, there was a clear suppression of intracellular TG turnover (Fig. 4.9), consistent with the proposed role for TGH in the lipolysis of intracellular stored TG.

It is likely that glucocorticoid regulation of enzymes involved in hepatic TG lipolysis and re-esterification had affected the balance between TG synthesis and lipolysis in such a way that an accumulation of intracellular TG occurred (Table 4.1). TG accumulation within the livers of dex-injected animals has been observed (27, 38, 47). Dex administration stimulates the synthesis of TG and increases the activity of several enzymes in the pathway for the *de novo* synthesis of TG. Dex is an activator of rat liver and lung fatty acid synthetases (21, 49, 50) and rat liver microsomal phosphatidate phosphohydrolase (51). Disruption of the gene for stearoyl-CoA desaturase-1 (SCD1) impaired de novo biosynthesis of hepatic TG and lowered the concentration of VLDL-TG in the plasma (ref). It is unknown whether or not dex regulates SCD1 expression, though the ratio of hepatic oleate/stearate, a measure of in vivo SCD activity, was elevated in dex-injected rats (ref). Therefore, it is likely that increased SCD activity contributes to increased TG biosynthesis. It is possible that inhibition of beta-oxidation may contribute to the accumulation of hepatic TG. Dex has not been demonstrated to affect mitochondria beta-oxidation, except in an instance where the concentrations of dex used exceeded the concentration used in the present study (52). Therefore it is seems that a combination of high TG synthesis and decreased TG lipolysis resulted in hepatic lipid accumulation.

A 2-fold higher plasma TG concentration was observed in the dex-injected mice when compared to controls (Table 4.1). Some groups have suggested that excess glucocorticoids stimulate TG secretion by the liver (53-55). Since TGH has been implicated in the provision of stored TG for VLDL secretion, the higher plasma

TG was not consistent with reduced TGH expression and activity. Nevertheless, when the hepatic TG secretion rate was measured in fasting mice following Triton WR-1339 injection, we did not observe any differences between the hepatic TG secretion rate of control or dex-injected mice (Table 4.2). Our observation is consistent with the results of several groups that reported no significant changes in the hepatic TG secretion rate in response to dex or cortisol (26, 38, 47, 56, 57). Furthermore, primary murine hepatocytes from dex-injected and control mice secreted similar quantities of TG mass into the media (Table 4.2), consistent with other studies (58, 59). Therefore, decreased clearance of circulating TG in the mouse likely resulted in the increased circulating TG concentration. Extracellular lipolysis is primarily controlled by lipoprotein lipase (LPL) an enzyme bound to the luminal side of the capillary endothelium. However, other lipases such as hepatic lipase also contribute to the clearance of TG from the circulation. LPL activity is greatest in adipose tissue (60) and several studies have demonstrated that endogenous (61, 62) or exogenous (38, 56, 63-65) glucocorticoid excess increased the plasma TG concentration and decreased adipose tissue LPL activity. It is possible that dex changed LDL-receptor activity. However, dex failed to affect LDL-receptor activity in rat hepatocytes (66) and HepG2 cells (67). Consequently, it seems unlikely that uptake of VLDL-TG through the LDL-receptor pathway is a contributing factor to the higher circulating TG observed in the plasma of dex-injected mice. Therefore decreased adipose tissue LPL activity is the likely cause of the elevated plasma TG concentration in our dex-injected mice and not increased hepatic secretion.

It has been shown that the pool of stored TG in the hepatocyte is not in simple equilibrium with VLDL-TG (3-5). The bulk addition of TG to the apoB100 particle represents the second stage of VLDL assembly that may be associated with the lipolytic mobilization of stored TG. In the dex-injected mouse, the turnover of the stored TG was suppressed (Fig. 4.9). Since the rate of hepatic TG secretion by dex-injected animals was at a similar level as controls, we suggest that a high level of *de novo* TG synthesis maintains TG secretion at a similar rate when utilization of stored TG for secretion is decreased. It appeared that the major effect of dex was to alter the balance between the utilization of stored TG versus *de novo* TG synthesis to supply TG for secretion. Since the rate of TG synthesis exceeded the rate of intracellular hepatic TG lipolysis as well as secretion, TG accumulated within the livers of dex-injected mice. Furthermore, the magnitude of hepatic TG accumulation caused by a similar dose and duration of dex injections is substantially higher in neonatal rats than in adult rats (47). Neonatal liver does not express TGH and consequently has a low capacity to mobilize stored TG for secretion (11, 19). When TG synthesis is increased by dex treatment, excess TG that accumulates in neonatal liver is not efficiently mobilized and consequently hepatic TG accumulates at a much higher level than adult liver.

It is possible that these observations are relevant to diurnal changes in hepatic lipid synthesis and secretion that are associated with periodicity of circulating glucocorticoid levels (68-73). For example, prior to a meal intake, glucocorticoid levels rise and this would result in a decrease in hepatic TGH levels and a

corresponding increase in lipogenic enzymes such as fatty acid synthase (49, 50), microsomal phosphatidate phosphohydrolase-1 (51, 74), DGAT-1 and -2 in the post-prandial period and a corresponding increase in hepatic lipid storage. Indeed, during the post-prandial period, the net synthesis of hepatic TG exceeds its disposal via secretion or oxidation during a period when circulating non-esterfied fatty acid and chylomicron levels are high (75). Then in the post-absorptive period, glucocorticoid levels fall and this would correspond to a decreased rate of TG synthesis and increased hepatic TGH levels which would be required to utilize stored TG. In the post-absorptive period, hepatic TG secreted in VLDL particles originated from the stored TG that had accumulated within the liver post-prandially (2). Individuals that have elevated levels of glucocorticoids during the post-absorptive period store higher levels of hepatic TG (76, 77). Whether diurnal changes in TGH expression correlate with diurnal changes in circulating glucocorticoids will be a subject of further investigation.

Interestingly, we identified a novel mode of TGH regulation. For the first time, we observed that TGH expression could be regulated in murine hepatocytes at the level of mRNA stability. Dex treatment of cells caused the TGH mRNA to be significantly reduced within 4h (Fig. 4.12B). However, it was observed that dex had minimal effects on nuclear run-on activity and TGH promoter-reporter luciferase activities (Fig. 4.10, 4.11). The murine TGH promoter has putative GRE half-sites, however the involvement of the GRE site in dex-mediated transcription is not evident as judged by the deletion mutant promoter-reporter analyses (Fig. 4.11). The murine TGH mRNA does however contain three AU-rich elements

(AREs) in its 3'-untranslated sequence (Fig. 4.13). AREs are involved in regulating the post-transcriptional stability of several mRNAs (78, 79). Proteins that bind to RNAs containing AREs can target susceptible mRNAs for cytosolic degradation through the exosome pathway (80). In particular, dex has been shown to decrease the stability of mRNAs that have AREs (81-83). The simultaneous treatment of hepatocytes with dex and actinomycin D did not cause the TGH mRNA level to drop to a similar level as in cells treated with dex alone. Hence, the synthesis of a factor that accelerates TGH mRNA degradation is transcriptionally dependent (Fig. 4.12C). The hypothesis that the AREs in the 3'-untranslated sequence of the TGH mRNA have a significant role in the stability of TGH transcripts will need to be formally tested with the use of the luciferase gene linked immediately upstream of the murine TGH 3'-untranslated sequence.

In conclusion, we have utilized the dexamethasone-injected mouse and primary hepatocyte models to study the regulation of the enzymes involved in hepatic microsomal TG lipolysis and re-esterification. TGH expression and activity was decreased while expression of DGAT was stimulated. The combination of reduced TG lipolysis and increased TG biosynthesis contributed to the accumulation of TG within the livers of dex-injected mice. We have observed that the rate of hepatic TG secretion was maintained at control levels. It appeared that the expression of the DGAT1 and 2 as well as other enzymes in the pathway for *de novo* TG synthesis (40) were stimulated and increased the proportion of secreted TG that was derived from *de novo* sources of TG when the utilization of TG stores for secretion was decreased. These results show that

during markedly increased TG synthesis, some TG are diverted entering the cytosolic storage pool and directly enter the luminal VLDL assembly pool. This observation may be relevant to diurnal variations in glucocorticoid levels and hepatic lipid levels observed during post-prandial and post-absorptive states.

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CHAPTER 5

FURTHER STUDIES ABOUT THE REGULATION AND FUNCTION IN ADIPOSE TISSUE OF THE MURINE TRIACYLGLYCEROL HYDROLASE

5.1 Introduction

White adipose tissue is quantitatively the most important tissue for the storage of TG which serves as a reservoir for excess energy. In times of energy need, lipolysis of stored TG from adipose tissue and fatty acids are released into the circulation to fulfill much of the energy requirements. As described in 1.5.1, hormone sensitive lipase (HSL) responds to acute hormonal signals to hydrolyze adipose tissue TG stores. However, HSL was not absolutely required for TG lipolysis in the adipose tissue from *Hsl*$^{-/-}$ mice. The targeted deletion of the HSL gene eliminated only CE hydrolysis and attenuated the hormonal stimulation of TG lipolysis, but reduced basal TG lipolysis only 50%, indicating that additional neutral TG lipase(s) are expressed in adipocytes (1, 2). At the moment, the only additional intracellular neutral TG lipase activity identified within the adipocyte is the microsomal TG hydrolase (TGH) (3). A role for TGH in the basal lipolytic activity and release of free fatty acids from adipose tissue was examined *in vitro* using 3T3-L1 adipocytes and *in vivo* using hamsters. A 40% decrease in circulating free fatty acids was observed in both 3T3-L1 adipocytes (4) and hamsters (5) treated with a TGH-specific inhibitor. A role was proposed for TGH in the insulin induced translocation of the glucose transporter isoform 4 (GLUT4) from intracellular membranes to the plasma membrane of rat adipocytes (6). It was suggested that TGH hydrolyzed acylglycerols or acyl-CoA esters to free fatty acids that are required for GLUT4 transport vesicle budding or fusion (6). However, we did not observe co-localization of TGH with GLUT4 vesicles as TGH was entirely localized to the

ER by confocal microscopy (4). Furthermore, inhibition of TGH did not affect the insulin induced translocation of GLUT4 to 3T3-L1 adipocyte plasma membranes (4). Therefore the proposed role for TGH in the insulin stimulated GLUT4 translocation was likely due to an artifact due to the permeabilization of rat adipocytes (6). Therefore, understanding the regulation of TGH in adipose tissue will represent a major advance towards a function for TGH and basal lipolysis (non-HSL mediated lipolysis) in the adipocyte.

As presented in Chapter 4, glucocorticoids have major effects on hepatic lipid metabolism and TGH expression is regulated by dex. The decreased TGH activity combined with increased TG biosynthesis resulted in the accumulation of hepatic TG. Greater visceral fat deposition is a feature of Cushing's syndrome and excess glucocorticoids result in obesity in mice (7). High levels of glucocorticoids are observed in most strains of obese rodents and adrenalectomy reduces the fat accumulation evident in obese rodents (8-10). Glucocorticoid replacement restored the accumulation of white fat in the adrenalectomized *ob/ob* mice, indicating that glucocorticoids play a permissive role in the development of obesity (9, 10). Therefore, glucocorticoids have major effects on adipose tissue lipid metabolism. In the present chapter, we attempt to extend these observations to several other conditions that are connected with the accumulation of hepatic TG and possible impaired TGH function. Consequently if glucocorticoids reduced TGH expression in adipose tissue in a similar manner as TGH was reduced in the liver, reduced TG lipolysis mediated by TGH could contribute to the accumulation of adipose tissue TG that is associated with excess glucocorticoids.

As described in 1.4.2, peroxisomal proliferator-activated receptors (PPAR) are nuclear hormone receptors that utilize poly-unsaturated fatty acids as ligands and regulate the expression of a range of genes involved in the expression of lipid metabolizing genes (11). Three related PPAR isotypes have been identified in vertebrates and were named PPARα, PPARδ and PPARγ. PPARα is expressed at the highest level in tissues that have a high capacity for mitochondrial and peroxisomal β-oxidation such as brown fat, liver, kidney and heart (12, 13). PPARα agonists have beneficial effects on the plasma lipid profile through the increased oxidation of fatty acids, decreased circulating TG and reduced hepatic and adipose tissue TG storage (14). PPARα null mice had lower levels of hepatic expression of several mitochondrial β-oxidative genes, compared to wild-type mice (15, 16). These changes are accompanied by hepatic lipid accumulation and an inability to increase ketone body production in response to fasting, suggesting that fatty acid oxidation was impaired in PPARα knock-out mice (15-20). Therefore, it is clear that PPARα is a critical mediator of lipid homeostasis. PPARγ on the other hand is primarily expressed in white adipose tissue. Overexpression of PPARγ promotes the conversion of pre-adipocytes into adipocytes (21) and even hepatocytes into adipocytes (22). A role for PPARγ in the induction of genes involved in the accumulation of lipids by adipocytes has been demonstrated (23). Thus, it is clear that PPARγ is a key regulator of adipogenesis.

As described in 1.4.3, leptin is a hormone that communicates energy status to the brain in order to control food intake and body

weight. Leptin also has direct effects on pancreatic cells, the liver and even adipocytes (24). The distribution of leptin receptors in these tissues has led to the recognition that leptin regulates energy balance separately from its effects on feeding behavior. Increasing leptin levels lead to increased fatty acid oxidation and a reduction in adipose tissue TG storage (25, 26). Leptin deficiency on the other hand is associated with an increase in fat deposition (27, 28).

Leptin acts in an autocrine-paracrine manner to inhibit lipogenesis through the suppression of acetyl-CoA carboxylase and fatty acid synthesis (29, 30). *ob/ob* mice have increased expression of enzymes that are rate limiting for fatty acid synthesis (27, 31). In addition to its suppression of lipogenesis, leptin stimulates lipolysis by adipocytes both *in vivo* and *in vitro* (30, 32-34). Interestingly, the mechanism by which centrally administered leptin leads to lipolysis and the loss of adipose tissue mass occurs without an increase in plasma free fatty acid (30). Several groups have demonstrated in cultured adipocytes that nanomolar quantities of leptin rapidly increased the rate of basal lipolysis, measured as glycerol release (30, 34-36). This observation was a consequence of the channelling of released FA towards mitochondria beta-oxidation (30, 37) and increased UCP2 expression (26, 38). Increased fatty acid oxidation was not restricted to adipose tissue and leptin treatment resulted in the reduction of TG stores in a variety of tissues (25, 26). Indeed leptin stimulated lipolysis and fatty acid oxidation in the liver of insulin resistant lipodystrophic mice, thereby improving the insulin sensitivity of these mice (39). The transcription factor PPARα is required for leptin to upregulate the β-oxidation of fatty acids, as in

the absence of PPARα, leptin does not reduce hepatic and adipose tissue TG stores (37).

In 1.5.1, the mechanism of hormone sensitive lipase mediated lipolysis was described. A rise in intracellular cAMP resulting from either adenylate cyclase activation or phophodiesterase inhibition stimulates TG lipolysis (40, 41). Leptin does not affect isoproterenol or forskolin induced lipolysis indicating that leptin did not stimulate lipolysis through a rise in intracellular cAMP (34, 36). Furthermore leptin did not affect HSL expression (37, 42). Consequently, leptin stimulated lipolysis may not involve activation of HSL. Leptin-stimulated lipolysis was not observed in obese rodents that lack a functional leptin receptor (30, 34-36). The action of leptin on adipose tissue was not mediated by neurotransmitters from the central nervous system as leptin continued to deplete TG from denervated white adipose tissue (43). In rabbits treated with leptin, it was shown that leptin stimulates a futile cycle of TG lipolysis and re-esterification and this serves to burn excess energy (44). Given that TGH is part of a lipolysis and re-esterification cycle in the liver, TGH could also be part of a similar cycle in adipose tissue as well. Regulation of TGH by leptin in adipose tissue could serve to modulate this futile cycle as well as the release of fatty acids. Therefore the function of TGH in adipose tissue was examined through investigating of TGH regulation in the differentiated adipocyte cell line 3T3-L1 as well as the adipose tissues and steatotic livers of genetically obese rodents.

5.2 Materials and Methods

5.2.1 *Reagents*

Restriction endonucleases, modifying enzymes, random primer labeling kit, Dulbecco's modified Eagle's medium (DMEM), sodium pyruvate, penicillin/streptomycin, fetal bovine and horse serum were obtained from Life Technologies Inc. Radioisotopes ([α-^{32}P]-dCTP, [γ-^{32}P]-dATP) were from Amersham Biotech. Western blotting reagents were obtained from Amersham Canada. Recombinant murine leptin was purchased from PeproTech Canada. Diethyl-*p*-nitrophenyl phosphate (E600) and all other chemicals were purchased from Sigma chemicals.

5.2.2 *Maintenance of animals*

Male C57/BL6 mice (6 weeks of age) were housed in a thermostatically controlled room with artificial lighting (12 hours light/12 hours dark). Animals had unrestricted access to water and to a commercial pelleted diet (22% protein/22% fat/56% carbohydrate). Mice were injected intraperitoneally with 40 mg/kg body weight dexamethasone (dex) delivered in 200 μl of corn oil, for 4 consecutive days and sacrificed 24h after the final injection, as described in a previous study (45). Control mice received corn oil only. Male PPARα KO mice (C57/BL6 background) (16) and wild-type mice (6 weeks of age) livers were removed and frozen in liquid nitrogen after being maintained on a standard lab diet or a diet containing 0.5% clofibrate for 14 days. Obese JCR:LA corpulent rats and lean littermates (4 months old) fed *ad libitum* the

commercial pelleted diet, were utilized (46). Obese *ob/ob* mice (47) and lean littermates (6 weeks of age) fed *ad libitum* the commercial pelleted diet, were utlized. Some obese mice received a 5 mg/kg body weight leptin injection delivered in sodium citrate for 2 consecutive days and were sacrificed 24h following the final injection. Control mice received only sodium citrate.

5.2.3 *PCR Primer Sequences*

All primers were synthesized at the DNA core facility, University of Alberta using a 394 DNA/RNA synthesizer (Applied Biosystems).

aP2F: 5'-GAACCTGGAAGCTTGTCTTCG-3'; aP2R: 5'-ACCAGCTT GTCACCATCTCG-3'; CYC1A: 5'-TCCAAAGACAGCAGAAAACTT TCG-3'; CYC2B: 5'- TCTTCTTGCTGGTCTTGCCATTCC-3'; DGAT1F: 5'-ATTCACGGATCATTGAGCG-3'; DGAT1R: 5'-CTGCCATGTCTGAGCATAGG-3'; DGAT2A: 5'- CTACGTTGGCTG GTAACTTCC-3'; DGAT2B: 5'- AACCAGATCAGCTCCATGG-3'; HSLF: 5'- ATGGATTTACGCACGATGACACAG-3'; HSLR: 5'-TAGCGTGACATACTCTTGCAGGAA -3'; Ex6F: 5'-CACTGCTGCT CTGATTACAACAG-3'; Ex10R: 5'-GCCTTCAGCGAGTGGATAGC-3'; X7F: 5'- CTTGCCTTTTTGGGAGAGCTG -3'; X10R: 5'-CTGCCACAGGATGGATGCAG -3'; P-TGHII: 5'- GAGCAAAGTTG GCCCAGTATTTCATCACCATTTTGCTGAG-3'

5.2.4 *Reverse transcription of RNA and quantitation of mRNA expression by real-time PCR*

Total RNA was reverse transcribed using an oligo dT_{20} primer and Superscript II reverse transcriptase (Invitrogen) according to the

manufacter's instructions. TGH (primers EX6F-EX10R), HSL (primers HSLF-HSLR), aP2 (primers aP2F-aP2R), DGAT2 (primers DGAT2A-DGAT2B) and cyclophilin (primers CYC1A-CYC2B) transcripts were detected by reverse transcription and followed by real time PCR using a LightCycler (Roche Diagnostics) machine. Reaction mixtures contained 0.5mM dNTPs, 3mM $MgCl_2$, 2.5μM of each primer, 1X SYBR Green I (Molecular Probes) in DMSO and 3U Taq polymerase in a total volume of 20 μl. Amplification was carried out as follows: 95°C, 57°C 10 s., 72°C 15 s. The number of cycles is indicated in the figure legends. Data analyses were performed using the LightCycler Software version 3.5 (Roche).

5.2.5 *RNA isolation and Northern analysis*

Total murine tissue RNA was isolated using Trizol Reagent (Life Technologies) according to the manufacturer's instructions. To perform Northern blot analysis, total RNA was separated in a 1% agarose/2.2M formaldehyde gel and transferred to a Hybond-N+ nylon membrane (Amersham) using a vacuum blotter (Biorad) according to manufacturer's instructions. Pre-hybridization and hybridization steps were carried out in 0.144M $NaHPO_4$, 7% SDS, 2μM $Na_4P_2O_7$, 2μM EDTA, 100μg ml^{-1} sheared salmon testes DNA (Sigma) and 1.5 x 10^6 cpm ml^{-1} at 55°C. For detection of TGH, ^{32}P-labelled 40bp oligonucleotide P-TGHII was utilized. The full length ^{32}P-labelled DGAT2 cDNAs was utilized for its detection by Northern analysis.

5.2.6 *Immunoblot analysis*

Mouse liver and adipose tissue homogenates (25μg of protein) were electrophoresed on an SDS/10% (w/v) polyacrylamide gel, transferred to a nitrocellulose membrane, and the expression of TGH was analysed by blotting with the anti-TGH polyclonal antibody as described previously (48). The expression of PDI was analysed by blotting with the anti-PDI polyclonal antibody (Santa Cruz) according to manufacturer's instructions.

5.2.7 *Lipase assay*

Lipolytic activity in murine liver microsomes was assessed using the fluorogenic substrate, 4-methylumbelliferyl heptanoate (MUH). A 1mM stock of MUH was prepared in tetrahydrofuran. The enzymatic reaction begins with the injection of 2.5μM MUH in 20mM Tris buffer, pH 8.0, 1mM EDTA, 300μM taurodeoxycholate to 1μg of microsomal protein in a 96 well plate (total volume 100μl). The plate is agitated at room temperature and read with a Fluoroskan Ascent FL Type 374 (Thermo Labsystems) in a kinetic fashion up to 10 min (excitation/emission wavelengths: 355/460nm). Data was analyzed using Ascent software version 2.4.2.

5.2.8 *Differentiation of 3T3-L1 fibroblasts into adipocytes.*

3T3-L1 fibroblasts (CL-173 American Type Culture Collection) were plated on 60mm dishes and permitted to reach confluence. Cells were differentiated into adipocytes according to the standard protocol (49). At confluence, differentiation medium was added to the cells: DMEM containing 10% fetal bovine serum (FBS), 0.25μM

dexamethasone, 3μg/ml insulin and 0.5mM isomethylbutylxanthine (IBMX). After three days, the differentiation medium was removed and cells were washed twice with phosphate buffered saline (PBS) and the medium was replaced with DMEM containing 10% FBS. Cells were given fresh media every three days thereafter. Additions to the medium at this point are indicated in the figure legends. Differentiation of 3T3-L1 cells into adipocytes was assessed by the morphological formation of large intracellular lipid droplets and expression of the adipocyte-specific fatty acid binding protein, aP2 (50).

5.2.9 *Glycerol Release Assay*

3T3-L1 fibroblasts were differentiated into adipocytes as described in 5.2.8. 24h following the replacement of the differentiation media with DMEM containing 10% FBS, the media was removed and replaced with DMEM in the absence of serum overnight (approximately 12h). Some cells are incubated overnight in the presence of 100μM E600 lipase inhibitor or 10μM of GR148672X TGH specific inhibitor (GWi) provided by Glaxo-Smith-Kline, or controls received vehicle alone. The DMEM was then replaced by Krebs-Ringer bicarbonate buffer (KRBB), pH 7.4, containing fatty acid free albumin (3.5 g/100ml) and 6mM glucose. Cells were incubated in the presence of inhibitors and hormones as indicated in the Figure legends for 4h. The media was removed and glycerol levels measured spectrophotometrically by a glycerol kinase reaction using the GPO-Trinder kit (Sigma). Cells were harvested and cellular TG levels were determined using the GPO-Trinder kit

(Sigma). The glycerol released into the media was normalized to cellular TG and expressed as a percentage of control (no hormones or inhibitors) ± standard deviation.

5.3 Results

5.3.1 *Dexamethasone suppresses the expression of TGH in adipose tissue*

Since dexamethasone (dex) reduced the expression of TGH in the liver, we examined whether dex regulated the expression of TGH in adipose tissue in a similar manner. TGH protein was significantly decreased ~80% by dex (Fig. 5.1). The effect of dex on TGH expression in the adipose tissue was even more pronounced than had been previously observed in the liver (Fig. 4.1, 4.5). Interestingly, DGAT2 expression was increased in a similar manner in the adipose tissue of dex-injected mice as it had been increased in the liver.

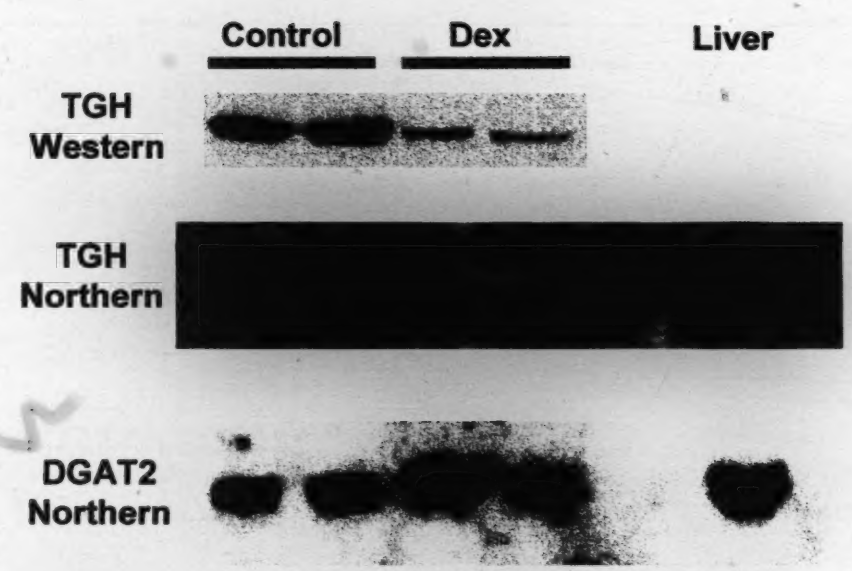

Fig. 5.1- Dexamethasone regulates expression of triacylglycerol Hydrolase (TGH) and diacylglycerol acyltransferase 2 (DGAT2) in the adipose tissue. Northern blotting was performed as described in 5.2.5. Western blotting was performed as described in 5.2.6. Murine TGH mRNA was detected by hybridization with a ^{32}P labeled TGH-specific 40-bp oligonucleotide probe, pTGHII. Murine DGAT2 mRNA was detected with the DGAT2 ^{32}P labeled cDNA probe.

5.3.2 *Differentiation dependent expression of TGH in 3T3-L1 adipocytes*

The expression of TGH was induced at day 4 following the induction of 3T3-L1 differentiation from fibroblasts into adipocytes (Fig. 5.2). This represents a later stage of 3T3-L1 cell differentiation into adipocytes and occurs when large intracellular lipid droplets begin to accumulate within the cytosol and later markers of differentiation, such as HSL and DGAT2 are expressed. Interestingly, we observed that dex regulated the expression of TGH mRNA in 3T3-L1 cultures, but only at a late stage of cell culture (8 days post-differentiation) (Fig. 5.2).

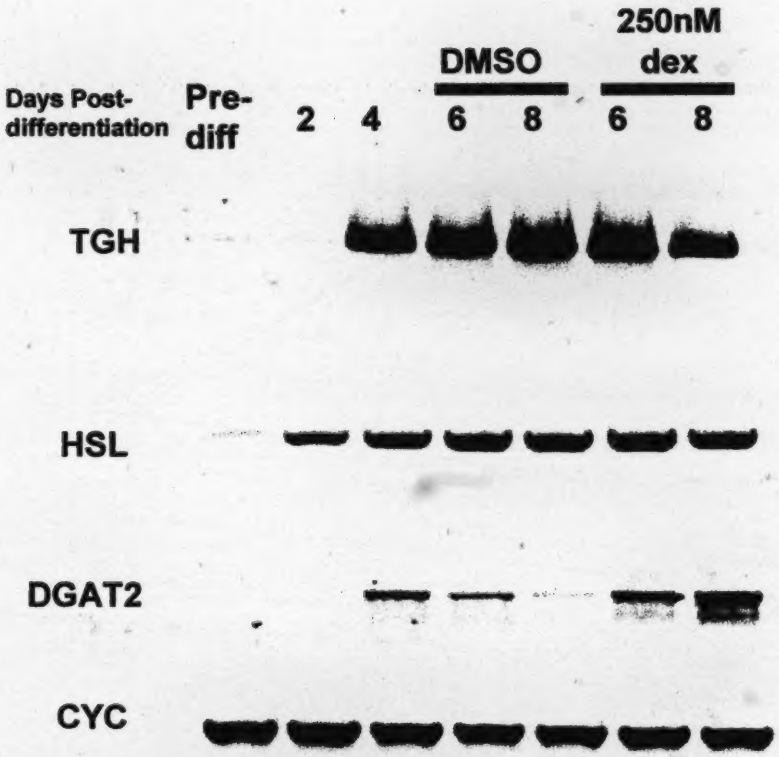

Fig. 5.2- TGH mRNA expression is induced 4 days following induction of 3T3-L1 adipocyte differentiation.
TGH, HSL, DGAT2 and cyclophilin (CYC) mRNA expression was determined by RT-PCR (22 cycles as described in 5.2.4. 3T3-L1 cells were differentiated as described in 5.2.8.

5.3.3 *Effect of peroxisomal proliferators on the expression of TGH*

Synthetic PPAR agonists specific for each of the three individual PPAR isoforms were provided by our collaborators at Glaxo-Smith-Kline. PPARγ ligands stimulate the adipogenic differentiation of 3T3-L1 cells (51). Significant changes in the expression of TGH were not observed when cells were incubated in the presence of 100nM of either PPARα, δ, or γ agonists for the indicated lengths of time (Fig. 5.3). However, when the expression of a marker of differentiation, the adipocyte specific fatty acid binding protein aP2 was examined, a significant induction of aP2 expression was not observed in the presence of the δ, or γ specific agonists (Fig. 5.3). This result indicated that the PPAR δ and γ agonists had not been effective in the differentiation of 3T3-L1 adipocytes in this experiment.

In order to determine whether PPARα regulated the expression of TGH in the liver, we utilized wild-type and PPARα$^{-/-}$ mice that had received clofibrate in their diet for 14 days. It appeared that clofibrate feeding increased the TGH mRNA in both wild-type and PPARα$^{-/-}$ mice, indicating a PPAR independent effect on TGH mRNA expression (Fig. 5.4). Furthermore, TGH mRNA expression was not significantly affected by the targeted deletion of the PPARα$^{-/-}$ gene, further supporting the idea that PPARα does not directly regulate TGH mRNA expression (Fig. 5.4). The TGH protein expression was not significantly affected by either clofibrate feeding or the targeted deletion of the PPARα$^{-/-}$ gene (Fig. 5.5). When the *in vitro* liver microsomal esterase activity was measured, no significant differences were observed between the esterase

activities of microsomes from either clofibrate fed mice or PPAR$\alpha^{-/-}$ mice compared to controls (Fig. 5.6). Since TGH accounts for the majority of microsomal esterase activity in the liver and TGH protein expression was also unchanged, one would not expect to observe a difference in the *in vitro* esterase activity of murine liver microsomes from clofibrate fed and PPAR$\alpha^{-/-}$ mice.

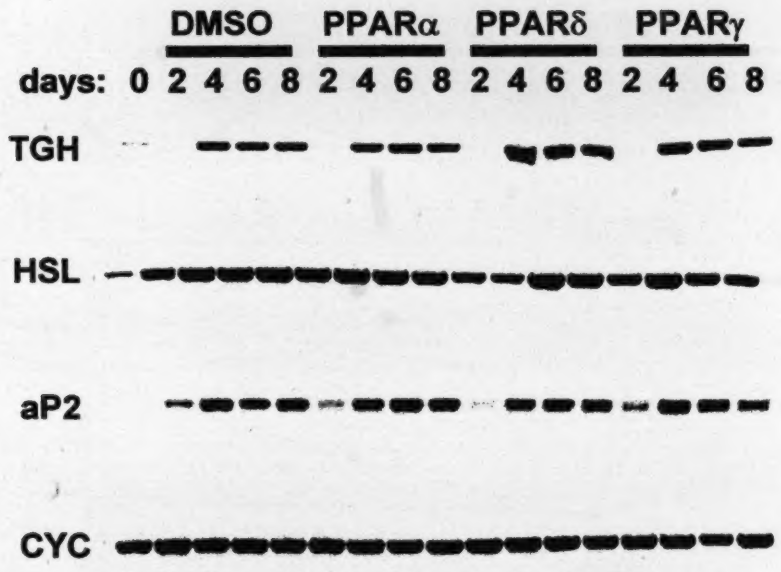

Fig. 5.3- No evidence for regulation of TGH mRNA expression by PPAR agonists in the 3T3-L1 cells.
TGH, HSL, aP2 and cyclophilin (CYC) mRNA ex-pression was determined by RT-PCR (22 cycles) as described in 5.2.4. 3T3-L1 cells were differentiated as described in 5.2.8. Cells were incubated in the presence of 100 nM of a PPAR-isoform specific agonist for the indicated days.

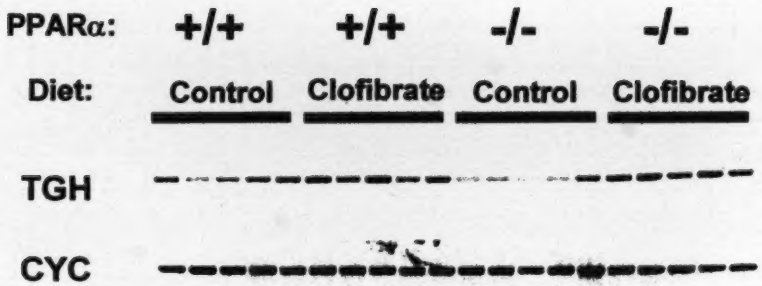

Fig. 5.4- TGH mRNA expression in the murine liver was indirectly increased by a PPARα agonist. Mice were fed 0.5% clofibrate in their diet for 14 days. TGH and cyclophilin mRNA expression was determined by RT-PCR (22 cycles) as described in 5.2.4

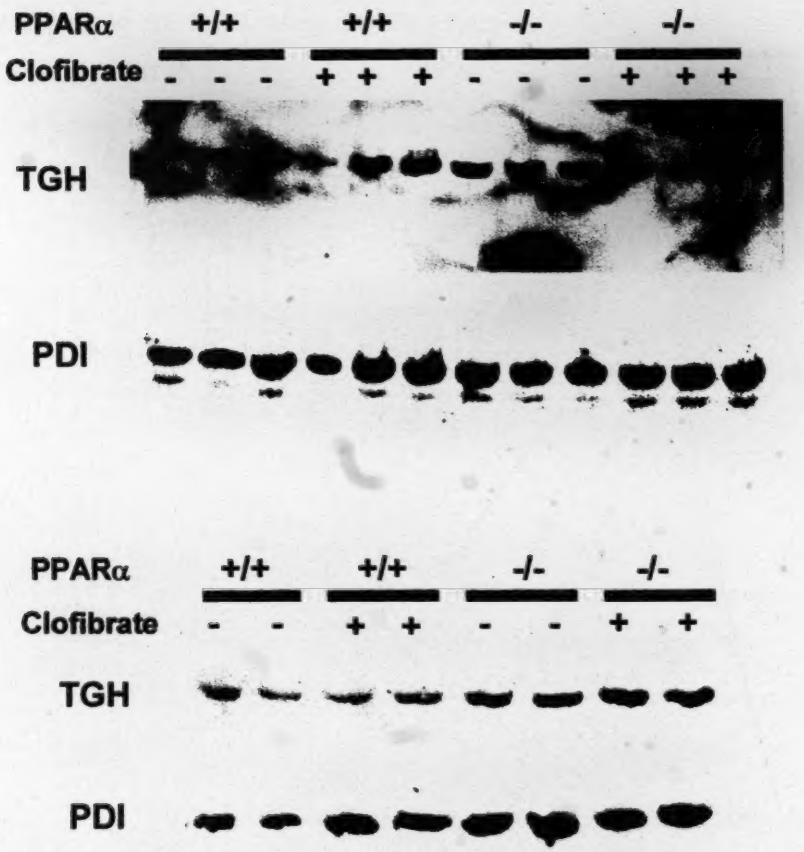

Fig. 5.5- PPARα does not affect TGH protein expression in murine liver microsomes. Mice were fed 0.5% clofibrate in their diet for 14 days. Western blotting was performed as described in 5.2.6. TGH and PDI proteins were detected using the respective anti-TGH and anti-PDI poly-clonal antibodies.

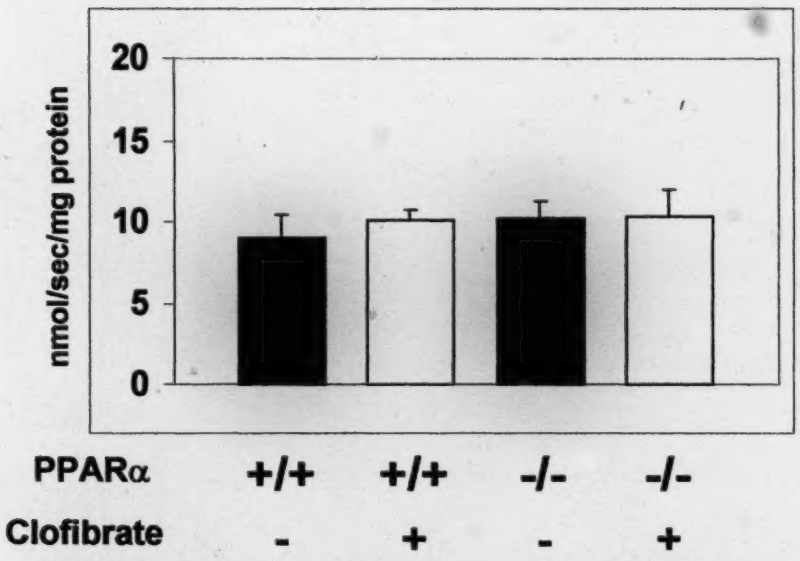

Fig. 5.6- PPARα does not affect the murine liver microsomal esterase activity. Liver microsomes were prepared from mouse livers harvested from wild-type and PPARα null mice. *In vitro* esterase activity was measured by the hydrolysis of 5-methylumbelliferylheptanoate as described in 5.2.7. The extent of hydrolysis was determined by measuring the excitation at 355nm and emission at 460nm in a kinetic fashion. n=5, average of three independent experiments.

5.3.4 *Reduced expression of TGH in genetically obese rodents*

JCR:LA corpulent rats are obese due to a mutation in their leptin receptors that causes the rats to become obese and diabetic (52). The livers of JCR:LA corpulent rats accumulate large quantities of TG (46). Interestingly, TGH expression was high in the livers of the lean rats, but was not detectable in the homogenates of the obese rats (Fig. 5.7). We extended our studies to include the *ob/ob* obese mouse that does not have circulating leptin (47). TGH expression was detectable in the livers of lean mice but was undetectable in the liver homogenates of obese mice (Fig. 5.8). Interestingly, 2 days of leptin replacement did not sufficiently restore TGH expression to the levels observed in the lean mouse (Fig. 5.8). TGH expression was also examined in adipose tissues from the same *ob/ob* mice. TGH expression was high in adipose tissue from lean mice and undetectable in the adipose tissue from obese mice. TGH expression was restored to the levels of the lean mouse by 2 days of leptin treatment in one mouse and remained undetectable in the second mouse (Fig. 5.9).

Fig. 5.7- TGH protein was not detectable in liver homogenates from obese JCR:LA corpulent rats. Western blotting was performed as described in 5.2.6. TGH and PDI proteins were detected using the respective anti-TGH and anti-PDI polyclonal antibodies.

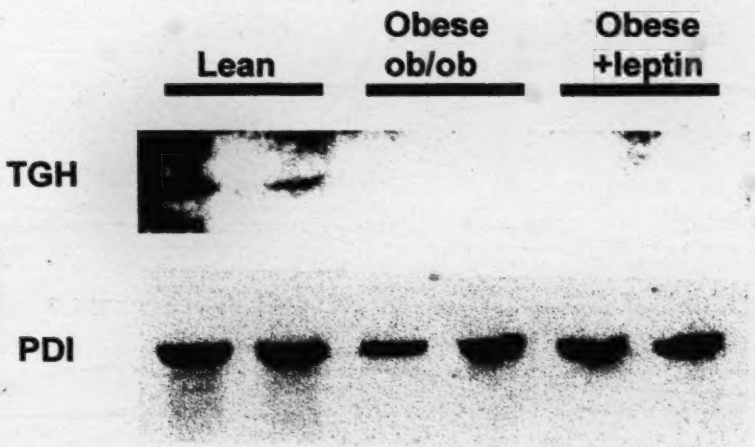

Fig. 5.8- TGH protein was not detectable in liver homogenates from obese *ob/ob* mice. Obese mice received a daily dose of 5μg leptin/g body weight for 2 days. Western blotting was performed as described in 5.2.6. TGH and PDI proteins were detected using the respective anti-TGH and anti-PDI polyclonal antibodies.

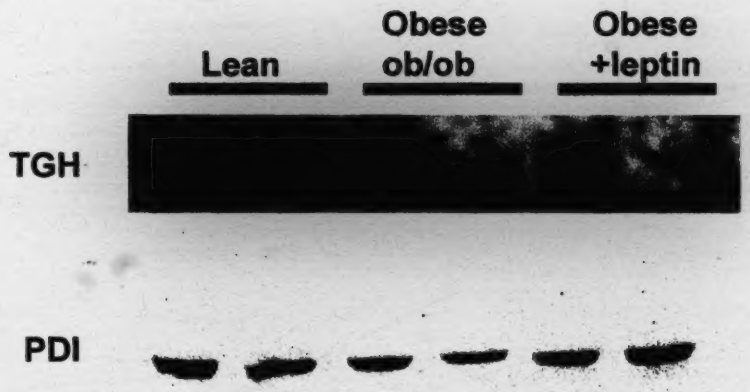

Fig. 5.9- TGH protein was reduced in adipose tissue homogenates from obese *ob/ob* mice. Obese mice Received a daily dose of 5μg leptin/g body weight for 2 days. Western blotting was performed as described in 5.2.6. TGH and PDI proteins were detected using the re-spective anti-TGH and anti-PDI polyclonal antibodies.

5.3.5 *Lipolytic inhibitors and glycerol release by 3T3-L1 adipocytes*

Leptin stimulates a novel form of lipolysis by adipocytes. Normally, upon lipolytic stimulation, adipocytes release glycerol and fatty acids into the media. However lipolytic stimulation of adipocytes by leptin results in the release of only glycerol into the media without the corresponding release of fatty acids (30). We investigated glycerol release into the media by 3T3-L1 adipocytes in response to leptin and the cAMP phosphodiesterase inhibitor, IBMX, which would activate hormone sensitive lipase. IBMX stimulated glycerol release 1.8-fold by 3T3-L1 adipocytes (Fig. 5.10). Leptin stimulated glycerol release by 1.5-fold (Fig. 5.10). In order to determine whether TGH had a role in leptin-stimulated lipolysis, 3T3-L1 adipocytes were pre-incubated with either a broad spectrum inhibitor of lipolysis, E600 or a TGH specific inhibitor provided by Glaxo-Smith-Kline (GWi). In control cells, E600 was much more effective at inhibiting glycerol release than the GWi (Fig. 5.10). In cells stimulated with leptin, both E600 and GWi inhibited lipolysis to levels observed in the control cells in the presence of the inhibitor (Fig. 5.10). In cells where IBMX was used to stimulate glycerol release, both E600 and GWi inhibited significant amounts of glycerol release, with E600 offering the most effective inhibition. However both inhibitors were less effective at inhibiting IBMX stimulated glycerol release than leptin stimulated glycerol release (Fig. 5.10).

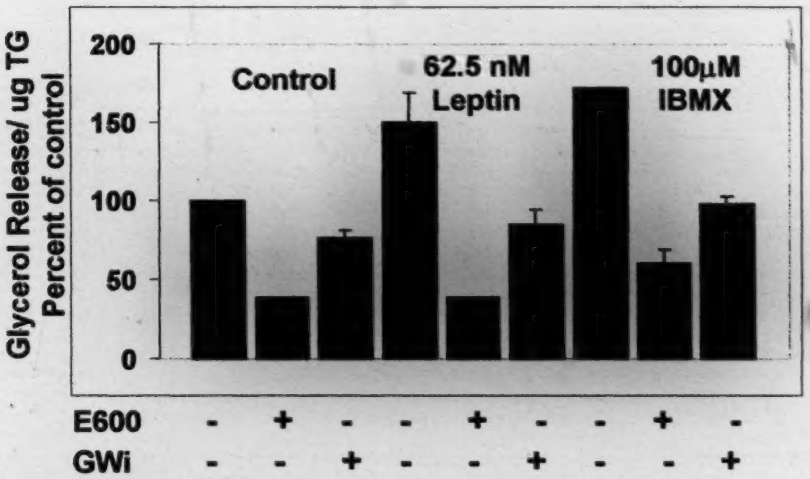

Fig. 5.10- Inhibition of leptin stimulated lipolysis by 3T3-L1 adipocytes. 3T3-L1 fibroblasts were differentiated into adipocytes as described in 5.2.8. The glycerol release assay was performed as described in 5.2.9. Some cells are incubated overnight in the presence of 100μM E600 and others in the presence of the 100nM GW inhibitor (GWi). DMEM was replaced by KRBB, pH 7.4 and cells were incubated in the pre-sence of inhibitors and either control (DMSO), 62.5nM leptin or 100μM IBMX for 4 hours and glycerol in media was measured. The glycerol released into the media was normalized to cellular TG and expressed as a percentage of control (no hormones or inhibitors) ± standard deviation.

5.3.6 *Direct effects of leptin on TGH expression in 3T3-L1 adipocytes*

Since it was possible that leptin had a direct effect on TGH expression, we measured the effect of leptin in 4h and 24h incubations with 3T3-L1 adipocytes. In both experiments, modest increases in TGH expression were observed with the greatest increase following 24h of leptin treatment (Fig. 5.11).

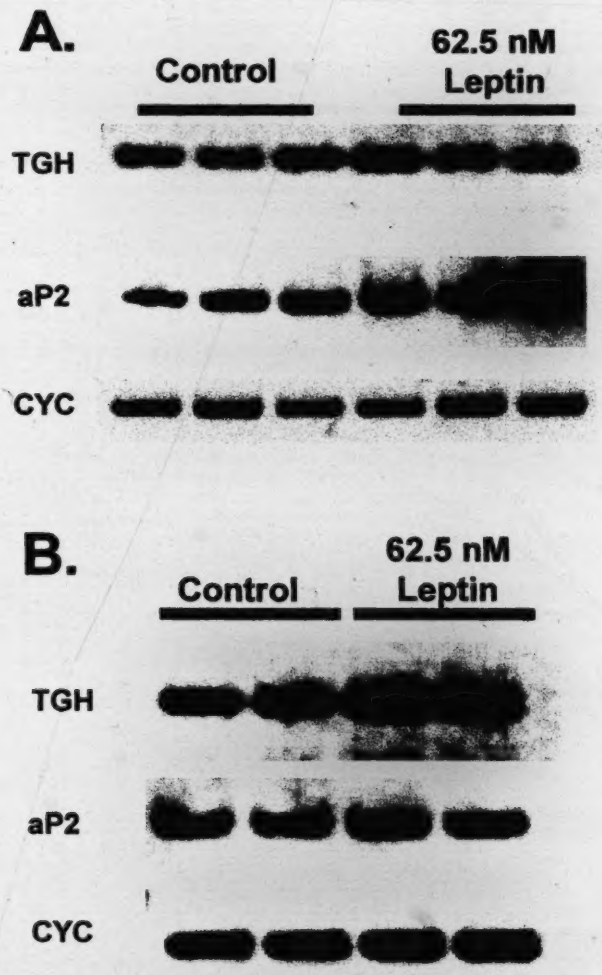

Fig. 5.11- Expression of TGH is modestly higher in leptin treated 3T3-L1 adipocyte cell cultures. Cells were differentiated as described in 5.2.8 and treated with 62.5nM leptin for either **A**. 4h or B. 24h. RNA was extracted and TGH, aP2 or CYC mRNA expression was determined by RT-PCR (22 cycles) as described in 5.2.4

5.4 Discussion

If TG biosynthesis exceeds TG lipolysis excess TG is stored within lipid droplets. If TG lipolysis exceeds TG biosynthesis, TG stored within the lipid droplet is depleted. This may have a beneficial effect if the released fatty acids are directed into oxidative pathways as opposed to the circulation. For example, mice lacking white fat have high circulating levels of fatty acids and accumulation of TG at higher levels than wild-type mice in peripheral tissues such as liver and muscle (53). These animals ultimately suffer from diabetes (53). We have utilized three separate mouse models that suffer from excess TG storage, in combination with 3T3-L1 adipocytes to examine whether TGH expression is impaired under these conditions. The dex-injected mouse had high levels of TG biosynthesis and accumulated high levels of adipose tissue TG. The JCR:LA corpulent rat (46) and *ob/ob* mouse (47) lack a functional leptin signalling system and accumulated massive quantities of adipose tissue and liver TG. In each of these obese animals, TGH expression was attenuated.

PPARα has a central role in the regulation of lipid metabolism. The role for PPARα in the up-regulation of enzymes necessary for fatty acid oxidation is well characterized (14-16). The PPARα agonist WY-14,643 reduces TG synthesis and apoB100 and B48 secretion as a large VLDL particle by rat hepatocytes (54). Instead, apoB100 and B48 were secreted as smaller denser lipoprotein particles. Therefore, PPARα directly regulates the size and TG load of VLDL particles. Smaller, less TG-rich lipopotein

particles promote the formation of LDL particles having shorter half-lives in the circulation than those generated from large TG-rich VLDLs (55). We hypothesized that the effects of PPARα on hepatic TG secretion may be mediated through an effect on TGH expression. A PPARα agonist also failed to affect TGH expression in the 3T3-L1 adipocyte cells (Fig. 5.3). Two weeks of clofibrate feeding to mice failed to affect the expression of TGH protein expression (Fig. 5.5) and *in vitro* esterase activity (Fig. 5.6). Furthermore, we utilized the PPARα KO mouse which accumulates high levels of hepatic TG due to impaired fatty acid oxidation and did not observe any changes in TGH expression (Fig. 5.5). Previously, it was observed that 5 weeks of exposure to WY-14,643 resulted in a modest reduction in the expression of rat liver carboxylesterases (56). Consequently, we conclude that PPARα cannot directly regulate TGH expression. The effects of PPARα on the density of hepatic VLDL particles that are secreted are primarily due to the ability of PPARα agonisits to reduce TG biosynthesis (54). Since TGH expression and *in vitro* esterase activity in wild-type mice, PPARα null mice and mice fed with clofibrate are similar, it is likely that the lipolysis of stored TG within the hepatocytes of these animals is similar. However, the differences between the hepatic TG levels and size of lipoprotein particles secreted are likely due to differences in the utilization of the released fatty acids. In PPARα null mice, secretion of hepatic TG is normal, but the portion of fatty acids directed into the oxidative pathway is reduced and in the absence of PPARα mediated down regulation of TG biosynthesis, hepatic TG accumulates. In mice fed clofibrate, TG

lipolysis is normal but it is likely that the lipolytic products are not resynthesized to form TG and consequently the hepatic TG stores are depleted. At the moment, it is unknown whether the DGAT isoforms are regulated by PPARα. In the clofibrate fed mice, the products of TG lipolysis are effectively directed towards the oxidative pathway.

Emerging molecular research suggests a direct role for leptin in partitioning fuel sources within adipose tissue and liver. Leptin decreases fatty acid synthesis in white adipose tissue and the liver and favours decreased tissue TG content and oxidation of fatty acids as fuel (57, 58). In the absence of leptin, TGH expression was not detected in either the liver or adipose tissue (Fig. 5.8, 5.9). Incubation of leptin in the media of 3T3-L1 adipocytes resulted in a modest increase in TGH expression (Fig. 5.11). These observations suggest that the leptin status of the animal affects the level of TGH expression.

Obesity results in an increased flux of fatty acids to the liver and increased TG synthesis. Since the availability of TG for incorporation into a VLDL particle determines the rate of hepatic TG secretion, it was assumed that the livers of *ob/ob* and *db/db* mice would produce VLDL at a higher rate. Instead, an accumulation of hepatic TG was observed with a decrease in TG secretion rate (59, 60). Therefore, fatty livers observed in these mice appeared to result from a missing step in the recruitment of TG for VLDL secretion. Our observations regarding the non-detectable expression of TGH in the livers of the *ob/ob* mice are important because they suggest that *ob/ob* mice have an impaired ability to mobilize stored TG resulting in the accumulation of TG in the liver.

The majority of the TG secreted by the *ob/ob* mice must be derived from the *de novo* synthesis of TG as the increased rate of TG synthesis would imply (57, 58).

In adipose tissue, TGH contributes to approximately 40% of the basal TG lipolysis as suggested by studies using the TGH inhibitor (1, 2) and the remaining lipolytic activity in adipose tissue isolated from HSL KO mice (4, 5). Therefore it is important to consider the regulation of TGH in adipose tissues. In rodents that synthesize higher levels of TG and accumulate larger white adipose tissue mass, including dex-injected mice (7-9), *ob/ob* mice (27, 28) and JCR:LA corpulent rats (46), TGH expression was reduced substantially. Therefore the absence of significant TGH activity would promote TG storage and even more so under conditions whereby TG biosynthesis has been increased.

Leptin stimulates a novel form of lipolysis in adipose tissue that does not involve free fatty acid release (30, 32-34). Leptin does not stimulate lipolysis by adipocytes derived from rodents that lack a functional leptin receptor (30, 34-36). Leptin stimulated lipolysis by 3T3-L1 adipocytes was measured in the presence of an inhibitor of TGH. It was observed that the TGH-specific inhibitor (GWi) was more effective at inhibition of leptin-stimulated lipolysis to levels observed in non-stimulated cells than IBMX-stimulated lipolysis. This result implied a direct role for TGH as a contributor to leptin-stimulated lipolysis (30, 32-34). Leptin does not affect the expression of HSL (37, 42) and does not stimulate lipolysis through modulation of intracellular cAMP levels that acutely regulate the activity of HSL (34, 36). Whether adipocytes derived from *Hsl*$^{-/-}$ mice remain responsive to leptin stimulated lipolysis is unknown. It

is unknown whether the activity of TGH responds to acute signals. Therefore we measured the effect of leptin on TGH expression in 3T3-L1 cells and observed a modest, albeit rapid increase in TGH expression (Fig. 5.11). Therefore it is possible that leptin can stimulate lipolysis through a direct effect on TGH expression. Alternatively, leptin may have a broader effect in the whole animal. In Chapter 4 and as observed in Fig. 5.1, dexamethasone reduces the expression of TGH. Given that leptin receptors are expressed in the adrenal glands (61, 62) and leptin reduces the synthesis and secretion of glucocorticoids by the adrenal glands (62, 63), leptin would have a positive, albeit indirect effect of maintaining a high level of TGH expression through the suppression of circulating glucocorticoid levels.

As described in 1.6.3 and 1.6.4 TGH is a part of a lipolysis/re-esterification cycle that is directly linked to TG secretion by the hepatocyte. If a lipolysis/re-esterification cycle exists in the adipocyte, a question exists as to its functional significance. TG turnover contributes to the stimulation of the basal metabolic rate during hyperleptinemia in rabbits (44). TG lipolysis/re-esterification represents an alternative mechanism in parallel with the known leptin induction of uncoupling proteins (37, 64, 65). The role of substrate cycling as a physiological furnace that increases energy expenditure by adipose tissue has been demonstrated following exercise and cold stress (66-69). A ubiquitous thermogenic mechanism would be leptin-sensitive as TGH appears to be. Adipocytes from obese humans generate less heat (70, 71) and have slower rates of TG turnover than adipocytes from lean subjects (72). Hormonal regulation of TG turnover may play a role in

determining the metabolic efficiency of the individual and their propensity towards obesity. Such changes in the lipolytic rate and fatty acid supply provide additional substrate necessary to support increased energy expenditure and a switch in oxidative fuel preference. Therefore we propose a role for TGH in adipose tissue basal energy use.

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CHAPTER 6

SUMMARY, CONCLUDING REMARKS AND FUTURE DIRECTIONS

In Chapter 1, the pathways of TG biosynthesis and their regulation are summarized. The proteins involved in TG storage, lipolysis and secretion are reviewed with special emphasis given to the newly identified triacylglycerol hydrolase (TGH) with respect to its function in the lipolysis of stored TG and the provision of stored TG for lipoprotein secretion.

Initially it was necessary to identify the murine TGH and its corresponding gene. Towards that goal, chapter 2 outlines the cloning of a novel murine cDNA encoding the murine TGH protein (1). Analysis of the murine TGH protein revealed its identity to the carboxylesterase gene family (2). The murine TGH protein was modeled based upon the X-ray crystal structure of a rabbit carboxylesterase (3). The model revealed that the structure of murine TGH was very similar to the structure of other lipases, revealing that TGH was also a member of the lipase super family (4). Furthermore, TGH shares a GXSXG catalytic serine motif and Ser, Glu, His catalytic triad in common with other known lipases. Consistent with this, expression of the murine TGH cDNA in McArdle RH7777 cells significantly increased esterase activity in cell homogenates (1). The expression profile of murine TGH was examined. Highest levels of TGH were observed in the liver, followed by heart, kidney and small intestine (1). The expression of TGH in tissues that secrete apoB-containing lipoprotein particles was consistent with a role for TGH in the provision of stored TG for lipoprotein secretion. In addition, it was discovered that TGH was expressed in adipose tissue, suggesting that TGH contributed to the proportion of basal TG lipolysis that did not involve HSL (5, 6).

In chapter 3, a novel murine gene encoding the TGH was

identified. This discovery permitted examination of the transcriptional regulation of the TGH gene as well as the design of a targeting vector to eliminate TGH expression in the intact mouse. Firstly, it was demonstrated that the TGH promoter sequence was functional and could drive the transcription of the luciferase reporter gene. Next, regulatory elements within the murine TGH promoter sequence were identified. Interestingly, it was discovered that binding of the Sp1 transcription factor to a GC-rich element within the murine TGH promoter was necessary to drive the transcription of the murine TGH gene (7). The binding of Sp1 to the murine TGH promoter was increased at the time of weaning when TGH expression is also induced (7). Therefore, in the subsequent chapters we studied the regulation of murine TGH expression further.

In Chapter 4, the regulation of TGH by the synthetic glucocorticoid, dexamethasone (dex), is considered. It was discovered that TGH expression was decreased approximately 40% in the livers of dex-injected mice compared to controls. Decreased TGH expression correlated well with TGH activity, measured both *in vitro* and in cultured hepatocytes, as determined by a dual-labeling pulse-chase experiment that measured intracellular TG turnover. Interestingly, the decreased TGH expression was a consequence of the regulation of TGH mRNA stability by dex. The dex-injected mouse had several interesting alterations in lipid metabolism. These included increased hepatic TG synthesis and storage without a corresponding increase in hepatic TG secretion. It appeared that the expression of the DGAT1 and 2 as well as other enzymes in the pathway for *de novo* TG synthesis (8) were stimulated and

increased the proportion of secreted TG that was derived from *de novo* sources of TG when the utilization of TG stores for secretion was decreased. This observation may be relevant to diurnal variations in glucocorticoid levels and hepatic lipid levels observed during post-prandial and post-absorptive states (9-14).

In Chapter 5, TGH expression in the adipose tissues of dex-injected mice was reduced more impressively than that observed in the liver. This result suggested that impaired TGH activity could have a role in the establishment of visceral adipose tissue accumulation observed when glucocorticoids are in excess (15). This observation led us to examine the expression of TGH in several rodent models of obesity (16, 17). Interestingly, TGH expression was not detectable in the livers from obese *ob/ob* mice nor the JCR:LA corpulent rat, as well as the adipose tissue from obese *ob/ob* mice.

Leptin reduces tissue TG storage as well as directly stimulates intracellular TG lipolysis (18-20). In cultured 3T3-L1 adipocytes, we observed that leptin marginally increased the expression of TGH, while a TGH inhibitor was more effective at inhibiting leptin stimulated lipolysis than IBMX induced lipolysis. Therefore, it appears that TGH could be regulated by leptin. It is well known that leptin acts directly on the adrenal gland to decrease glucocorticoid synthesis and secretion (21, 22). Since we established in chapter 4 that glucocorticoids suppressed TGH expression, leptin could act indirectly to maintain TGH expression at a high level by promoting lower levels of circulating glucocorticoids. Leptin is also an insulin sensitizing hormone that can act on pancreatic beta-cells to inhibit insulin secretion (23-26). Obese

rodents that lack the leptin signalling system are hyperinsulinemic, insulin resistant and ultimately become diabetic (16, 17). It is unknown whether insulin can directly regulate TGH expression, though a recent report showed decreased TGH expression in the livers of mice with tumour necrosis factor-α induced insulin resistance (27). Furthermore, carbohydrates appeared to be a positive regulator of TGH expression as rats fed diets enriched in fructose and glucose increased the TGH expression 2-fold, MTP expression 3-fold and increased apoB48 and liver TG levels (Lehner, R., personal communication, 2003). Taken together, these results suggest that the insulin and leptin signals are positive regulators of TGH expression.

The regulation of TGH through the PPAR pathway was examined utilizing PPAR-isoform specific agonists on 3T3-L1 adipocytes, clofibrate feeding of mice and PPARα null mice. Evidence for the regulation of TGH expression by PPARs was not observed. Furthermore, feeding mice diets enriched in saturated, monounsaturated and unsaturated fats in combination with or without cholesterol did not exhibit changes in TGH expression (Lehner, R., personal communication, 2003). Taken together, these results conclusively demonstrate that fatty acids do not regulate TGH expression.

To summarize the findings of these studies about TGH regulation, some common trends emerge. Leptin and PPARα promote fatty acid oxidation and decreased TG synthesis and TG storage and TGH expression was high. In the absence of leptin and in the presence of excess glucocorticoids TG synthesis and storage

was high and TGH expression was low. A confounding factor is the increased TG storage despite normal TGH expression in PPARα null mice. However, TGH is placed at the first stage in the removal of fatty acids from the TG storage droplet. If the oxidative pathway is not upregulated sufficiently, mobilized fatty acids are simply re-esterified and secreted from the cell or perhaps to a greater extent, re-esterified and returned to the TG storage droplet as appears to be the case in PPARα null mice. However, when TGH expression was impaired, as was the case in dex-injected mice as well as obese *ob/ob* mice and JCR:LA corpulent rats, we also observe that the mobilization of stored TG is likely impaired and consequently lipolytic products cannot be supplied for either oxidation or secretion. When combined with increased TG biosynthesis, as is observed in these conditions, the result is a massive accumulation of intracellular TG.

These observations are important in light of two mouse models we are presently creating in our laboratory. The targeted deletion of the TGH gene may be analogous to the situation observed in leptin deficient rodents, as long as the expression of other carboxylesterases are not upregulated to compensate for the lost TGH expression (see chapter 3). The livers of TGH null mice would be expected to be impaired in their ability to mobilize TG from the storage pool and consequently VLDL-TG concentrations would be lower as long as the *de novo* synthesis of TG was not increased as a compensatory mechanism. If *de novo* TG biosynthesis is maintained at a low level, the hepatic TG accumulation could be manageable, however hepatic steatosis would likely result if animals

were challenged with a high fat diet. In adipose tissue, the targeted deletion of TGH would not severely impair TG lipolysis due to the presence of TGH, though leptin stimulated lipolysis may be impaired. However, lower levels of VLDL secretion would imply lower levels of adipose tissue mass and consequently a leaner mouse, unlike the leptin deficient rodents.

A TGH transgenic mouse over-expressing the human TGH cDNA is also presently being created. Higher levels of TGH would imply depletion of hepatic TG stores. If lipolytic products are channelled into the secretory pathway, TGH over-expression would result in increased VLDL secretión, as observed when TGH was over-expressed in McArdle RH7777 cells (28). Conversely, the lipolytic products could simply be recycled to the storage pool without a corresponding change in hepatic TG levels. If VLDL secretion is increased, this would imply increased VLDL in the circulation and if adipose tissue lipoprotein lipase can sufficiently remove the increased quantity of VLDL from the circulation, an increased storage of TG within the adipose tissue would result. However, if this approach also involves increased TGH expression in adipose tissue, one would expect increased basal free fatty acid release from adipose tissue resulting in an increased flux of fatty acids back to the liver. This would imply high levels of lipid in the circulation and possibly an atherogenic lipoprotein profile.

In summary, these studies represent a major advance in our understanding of the function and regulation of the murine microsomal triacylglycerol hydrolase.

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